



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>4</sup> :</b> A61K 39/005, 39/04, 39/40 C12N 15/00, 1/00, C12P 21/00 G01N 33/53, A61K 39/395	<b>A1</b>	<b>(11) International Publication Number:</b> WO 90/02564 <b>(43) International Publication Date:</b> 22 March 1990 (22.03.90)
<b>(21) International Application Number:</b> PCT/US89/03955 <b>(22) International Filing Date:</b> 12 September 1989 (12.09.89)  <b>(30) Priority data:</b> 243,474 12 September 1988 (12.09.88) US  <b>(60) Parent Application or Grant</b> (63) Related by Continuation US 243,474 (CIP) Filed on 12 September 1988 (12.09.88)  <b>(71) Applicant (for all designated States except US):</b> CODON [US/US]; 213 East Grand Avenue, South San Francisco, CA 94080 (US).  <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only) :</b> DRAGON, Elizabeth [US/US]; 42 Park Lane, Orinda, CA 94563 (US). FAULDS, Daryl [US/US]; 1345 Hillcrest Boulevard, Millbrae, CA 94030 (US). SIAS, Stacey [US/US]; 37 Carlson Court, San Anselmo, CA 94960 (US).		<b>(74) Agent:</b> WEBER, Kenneth, A.; Townsend and Townsend, One Market Plaza, 2000 Steuart Tower, San Francisco, CA 94105 (US).  <b>(81) Designated States:</b> AT (European patent), BE (European patent), CH (European patent), DE (European patent), FR (European patent), GB (European patent), IT (European patent), LU (European patent), NL (European patent), SE (European patent), US.  <b>Published</b> <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
<b>(54) Title:</b> VACCINE DIAGNOSTIC EMPLOYING PROTEINS HOMOLOGOUS TO HEAT SHOCK PROTEINS OF TRYPANOSOMA CRUZI  <b>(57) Abstract</b>  This invention relates to vaccines and diagnostics and more particularly to vaccines and diagnostics which employ proteins and/or fragments and/or derivatives thereof having homology to heat shock proteins of Trypanosoma cruzi.		

***FOR THE PURPOSES OF INFORMATION ONLY***

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	ES	Spain	MG	Madagascar
AU	Australia	FI	Finland	ML	Mali
BB	Barbados	FR	France	MR	Mauritania
BE	Belgium	GA	Gabon	MW	Malawi
BF	Burkina Faso	GB	United Kingdom	NL	Netherlands
BG	Bulgaria	HU	Hungary	NO	Norway
BJ	Benin	IT	Italy	RO	Romania
BR	Brazil	JP	Japan	SD	Sudan
CA	Canada	KP	Democratic People's Republic of Korea	SE	Sweden
CF	Central African Republic	KR	Republic of Korea	SN	Senegal
CG	Congo	LI	Liechtenstein	SU	Soviet Union
CH	Switzerland	LK	Sri Lanka	TD	Chad
CM	Cameroon	LU	Luxembourg	TG	Togo
DE	Germany, Federal Republic of	MC	Monaco	US	United States of America
DK	Denmark				

VACCINE DIAGNOSTIC EMPLOYING PROTEINS HOMOLOGOUS  
TO HEAT SHOCK PROTEINS OF TRYPANOSOMA CRUZI

BACKGROUND OF THE INVENTION

5           This invention relates to vaccines and  
diagnostics and more particularly to vaccines and  
diagnostics which employ proteins and/or fragments  
and/or derivatives thereof having homology to heat  
shock proteins of *Trypanosoma cruzi*.

10           Heat shock proteins, sometimes referred to as  
stress proteins, have been found in a wide variety of  
cells, and have been generally described in an  
article written by Tissieres on pages 419 through 429  
of "Heat Shock from Bacteria to Man" (Cold Spring  
15   Harbour Laboratory, 1982).

DESCRIPTION OF THE FIGURES

Figure 1 provides the gene and derived amino  
acid sequence for the Hsp70 antigen of *T. cruzi*.

20           Figure 2 provides an alignment of heat shock  
proteins from a variety of organisms: 1. *M. hyo-*  
*pneumoniae*, 2. *Bacillus megaterium*, 3. *Escherichia*  
*coli*, 4. *T. cruzi*, 5. *T. cruzi*, 6. Rat, 7. *Xenopus*  
*laevis* 8. human, 9. chicken, 10. *Zea mays*, 11. *Serratia*  
*marcescens*.

25           Figure 3 provides a restriction map of  
pMYCO16 containing the full length gene for the Hsp70  
antigen of *M. hyopneumoniae*.

30           Figure 4 provides an intermediate plasmid for  
the expression of the Hsp70 antigen of *M.*  
*hyopneumoniae*.

Figure 5 provides the gene and derived amino  
acid sequence for the Hsp70 antigen of *M.*  
*hyopneumoniae*.

35           Figure 6 provides restriction map of pMYCO29  
which is a low level expression plasmid containing the

full length gene for the Hsp70 antigen of *M. hyopneumoniae*.

Figure 7 provides a restriction map of pMYC031 which is a high level expression plasmid containing the full length gene for the Hsp70 antigen of *M. hyopneumoniae*.

Figure 8 provides a restriction map of pCAM101 containing the trpT176 gene.

Figure 9 provides a restriction map of pMYC032 which is an expression plasmid containing the full length gene for the Hsp70 antigen of *M. hyopneumoniae* and the trpT176 gene.

Figure 10 provides a restriction map of pMGA4 which is an expression plasmid containing the full length gene for the Hsp70 antigen of *M. gallisepticum*.

Figure 11 provides the gene and derived amino acid sequence for the Hsp70 antigen of *M. hyopneumoniae*.

Figure 12 provides a restriction map of pMGA10 which is an expression plasmid containing the full length gene for the Hsp70 antigen of *M. hyopneumoniae* and the trpT176 gene.

#### SUMMARY OF THE INVENTION

Vaccines are disclosed for the protection against organisms which comprise a physiologically acceptable carrier with a protein which is capable of eliciting an antibody which recognizes at least one epitope of a native protein present in the organism, the native protein having at least 50% homology with a heat shock protein of *T. cruzi*. Processes for protecting a host against an organism are also disclosed which comprise administering an effective amount of a protein capable of eliciting an antibody which recognizes at least one epitope of a native protein present in the organism, the native protein

having at least 50% homology with a *T. cruzi* heat shock protein.

Further disclosed are processes for determining an organism in a host which comprise  
5 contacting a sample derived from a host containing an organism or suspected of containing an organism with an antibody or antibody fragment which recognizes at least one epitope of a native protein present in the organism, the native protein having at least 50%  
10 homology with a heat shock protein of *T. cruzi*; and determining protein present in the organism bound to the antibody.

For such vaccines and processes, the native protein referred to above may be derived from a species  
15 of *Mycoplasma*, *Mycobacteria* or *Trypanosoma*, provided that the native protein is not derived from *Trypanosoma cruzi*. Preferably, the native protein of *Mycoplasma* derivation is one selected from the group consisting of *M. mycoides*, *M. bovis*, *M. bovis genitalium*, *M. bovoculi*,  
20 *M. bovirhinis*, *M. dispar*, *M. hyorhinis*, *M. hyosynoviae*, *M. hyopneumoniae*, *M. gallisepticum*, *M. pneumoniae*, and *M. synoviae*, most preferably from *M. hyopneumoniae* and *M. gallisepticum*. The native protein of *Mycobacteria* derivation is preferably one selected from the group  
25 consisting of *M. bovis*, *M. leprae*, and *M. tuberculosis*.

The recombinant sequence of nucleic acid encoding the heat shock proteins of *M. hyopneumoniae* and *M. gallisepticum* is also disclosed.

#### DETAILED DESCRIPTION

30 Applicant has found that certain heat shock proteins and/or fragments and/or derivatives thereof may be employed in a vaccine to protect against an organism containing such heat shock protein.

Applicant has further found that certain heat  
35 shock proteins and/or fragments or derivatives thereof, as well as antibodies produced in response

to such heat shock proteins and/or fragments or derivatives thereof may be employed as a diagnostic for determining an organism containing such heat shock proteins.

5           Applicant has also found that certain DNA (RNA) sequences encoding for a heat shock protein of an organism may be employed as a diagnostic for determining the organism.

10           In accordance with the one aspect of the present invention, there is provided a vaccine for protecting against an organism which includes a heat shock protein wherein the vaccine includes a protein capable of eliciting an antibody which recognizes at least one epitope of a heat shock protein of the  
15           organism which heat shock protein of the organism has at least 50% homology with a heat shock protein of *Trypanosoma cruzi* (T. cruzi).

          In accordance with another aspect of the present invention, there is provided a process for  
20           protecting against a disease caused by an organism which includes a heat shock protein by administering to a host at least one protein capable of eliciting an antibody which recognizes at least one epitope of a heat shock protein of the organism which heat shock  
25           protein of the organism has at least 50% homology with a heat shock protein of *Trypanosoma cruzi* (T. cruzi).

          The term that an antigen or protein has at least 50% homology with a heat shock protein of T.  
30           cruzi, as used herein, means that on a position by position basis, at least 50% of the amino acids of the heat shock protein of T. cruzi are also present in the antigen or protein.

          More particularly, in a preferred embodiment  
35           the heat shock protein or polypeptide of T. cruzi with which an antigen or protein is to have at least 50% homology is at least one of the T. cruzi heat shock

proteins having a molecular weight of about 70 kD, or about 85 kD or about 65 kD, preferably the heat shock protein having a molecular weight of about 70 kD.

5       The *T. cruzi* heat shock protein having a molecular weight of about 70 kD may be prepared as described in Example 1. The amino acid and DNA sequence for the 70 kD protein is shown in Figure 1 of the drawings, with the 70 kD protein starting at base pair 25 and terminating at base pair 677.

10       The *T. cruzi* heat shock protein having a molecular weight of about 85 kD is described by Dragon et al. Molecular and Cellular Biology, Volume 7 No. 3 Pages 1271-75 (March 1987).

15       The protein which is present in the organism and which is at least 50% homologous to a *T. cruzi* heat shock protein will sometimes be referred to herein as the "homologous protein" or the "homologous heat shock protein".

20       The protein employed in formulating the vaccine for protection against an organism may be identical to a homologous protein present in the organism to be protected against, or may be a fragment or derivative of such homologous protein, provided that the protein which is used in the vaccine is capable of  
25       eliciting an antibody which recognizes at least one epitope of the homologous protein. For example, the protein employed in the vaccine may be only a portion of the homologous protein present in the organism or may have one or more amino acids which differ from the  
30       amino acids of the homologous protein in the organism or may be the homologous protein (or fragment or derivative thereof) fused to another protein.

35       The term "protein which is capable of eliciting an antibody which recognizes at least one epitope of a native protein present in the organism, said native protein having at least 50% homology with a heat shock protein of *T. cruzi*" (such protein present

in the organism is what is sometimes referred to as the homologous protein) encompasses the homologous protein present in the organism or a fragment of such homologous protein or a derivative of such homologous protein or a fusion product of such homologous protein (or fragment or derivative thereof) with another protein. As should be apparent, the protein or proteins included in the vaccine may include more or less amino acids or amino acids different from the amino acids of the homologous protein present in the organism.

The protein or proteins employed in the vaccine may be identified and produced by recombinant techniques. More particularly, the DNA (or RNA) encoding for a *T. cruzi* heat shock protein is employed as a probe to identify DNA present in the organism against which protection is sought which has at least 50% homology with the DNA (RNA) encoding for a *T. cruzi* heat shock protein. The DNA of the organism having the requisite homology is sometimes referred to herein as the "homologous DNA".

The homologous DNA of the organism identified by such probe is employed to produce homologous protein of the organism by recombinant techniques. Thus, for example, the DNA encoding for the protein of Figure 1 may be suitably labeled, for example with <sup>32</sup>P, by procedures known in the art to thereby provide a probe for identifying DNA in the organism having at least 50% homology with the DNA sequence encoding for the protein of Figure 1.

Figure 2 presents an alignment of the amino acid sequences of Hsp70 proteins from a number of species. The amino acids are depicted by their single letter abbreviations. Stretches of sequence identical in all examined species were identified (denoted by upper case text in the consensus sequence depicted below the individual sequences). Several regions



containing sequences at least six amino acids in length which were identical in all Hsp70 sequences. For example, between amino acid 138 and 209 of *T. cruzi* lie the sequences TVPAYF, RIINEPTA, and DLGGGTFD which are conserved in Hsp70 sequences. The DNA sequences which could encode these conserved sequences were determined. The 17-mer nucleotide sequences having low coding degeneracy serve as universal oligonucleotide probes for Hsp70 genes.

10           The probing conditions selected are such that hybrids are identified in which there is at least 50% homology between the selected DNA probe which encodes for a *T. cruzi* heat shock protein and the DNA being probed for in the organism. Such probing is done at  
15           relatively low stringency. Low stringency is achieved by known methods such as reduced temperature and increased salt concentrations (e.g., hybridizing at 37°C and 5-6 X standard salt-citrate buffer or 5-6X standard salt-EDTA-Tris buffer).

20           The selected homologous DNA of the organism may be included in any of a wide variety of vectors or plasmids for producing a protein to be employed in formulating a vaccine against the organism. Such vectors include chromosomal, nonchromosomal and  
25           synthetic DNA sequences; e.g., derivatives of SV40; bacterial plasmids; phage DNA's; yeast plasmids; vectors derived from combinations of plasmids and phage DNAs, viral DNA such as vaccinia, adenovirus, fowl pox, virus, pseudorabies, etc.

30           The appropriate DNA sequences may be inserted into the vector by a variety of procedures. In general, the DNA sequences are inserted into an appropriate restriction endonuclease site by  
procedures known in the art. Such procedures and  
35           others are deemed to be within the scope of those skilled in the art.

The DNA sequences in the vector are operatively linked to an appropriate expression control sequence(s) (promoter) to direct mRNA synthesis. As representative examples of such promoters, there may be mentioned: LTR or SV40 promoter, the E. coli lac or trp, the phage lambda PL promoter and other promoters known to control expression of genes in prokaryotic and eukaryotic cells or their viruses.

The expression vector also contains a ribosome binding site for translation initiation and a transcription terminator. The vector may also include appropriate sequences for amplifying expression.

In addition, the expression vectors preferably contain a gene to provide a phenotypic trait for selection of transformed host cells such as dihydrofolate reductase or neomycin resistance for eukaryotic cell culture, or such as tetracycline or ampicillin resistance in E. coli.

The vector containing the appropriate DNA sequences as hereinabove described, as well as an appropriate promoter or control sequence, may be employed to transform an appropriate host to permit the host to express the protein. As representative examples of appropriate hosts, there may be mentioned: bacterial cells, such as E. coli, Salmonella typhimurium, fungal cells, such as yeast; animal cells such as CHO or Bowes melanoma; plant cells, etc. The selection of an appropriate host is deemed to be within the scope of those skilled in the art from the teachings herein.

The expression vehicle including the appropriate DNA sequences for the protein to be expressed and the t-RNA inserted at the selected site may include a DNA or gene sequence which is not part of the gene coding for the protein. For example, the desired DNA sequence may be fused in the same reading

frame to a DNA sequence which aids in expression or improves purification or permits increases in the immunonogenicity.

In employing recombinant techniques for  
5 producing the active protein, purifications, digestions, ligations and transformations may be accomplished as described in "Molecular Cloning: A Laboratory Manual" by Maniatis et al., Cole Spring Laboratory, 1982 ("Maniatis"). In addition,  
10 transformations may be accomplished by the procedure of Cohen, PNAS, 69:2110 (1973).

When seeking to develop a vaccine, neutralizing or protective antibodies could be targeted toward discontinuous, conformation-dependent epitopes  
15 of the native antigen. One must therefore consider whether the protein obtained from the recombinant expression system might have a three dimensional structure (conformation) which differs substantially from that of the original protein molecule in its  
20 natural environment. Thus, depending on the immunogenic properties of the isolated proteins, one might need to renature it to restore the appropriate molecular conformation. Numerous methods for renaturation of proteins can be found in the scientific  
25 literature and include; 1) denaturation (unfolding) of improperly folded proteins using agents such as alkali, chaotropic agent, organic solvents, and ionic detergents followed by a renaturation step achieved by dilution, dialysis, or pH adjustment to remove the  
30 denaturant, and 2) reconstitution of proteins into a lipid bilayer or liposome to re-create a membrane like environment for the immunogenic protein.

The vaccine which includes a protein of the type hereinabove described may be employed in a vaccine  
35 for protecting against diseases caused by a wide variety of organisms. Table 1 provides representative examples of such organisms. Of particular interest are

species of Trypanosoma, Mycoplasma and Mycobacteria. Trypanosoma and Mycoplasma heat shock proteins are described herein. Heat shock proteins for Mycobacteria are known. Young et al., P.N.A.S. (USA), 85:4267-4270  
5 (1988).

A host may be protected against a disease caused by a certain organism by incorporating into the vaccine a protein which is capable of eliciting antibodies which are recognized by at least one  
10 epitope of a homologous protein of the organism. As hereinabove indicated the protein which is capable of eliciting such antibodies (hereinafter sometimes referred to as the active protein) may correspond to the homologous protein of the organism or may be a  
15 fragment or derivative thereof. As should be apparent, if the disease against which a host is to be protected is Chagas, which is caused by T.cruzi, the protein which is included in the vaccine would be one or more heat shock proteins of T. cruzi or a fragment or  
20 derivative thereof capable of eliciting antibodies which recognize an epitope of T. cruzi heat shock protein. The host which is protected is dependent upon the organism against which protection is sought. In general, the host is an animal (either a human or  
25 nonhuman animal) which is subject to a disease caused by the organism. Thus, for example if the organism against which protection is sought is one which is known to cause disease in man, then the vaccine including the active protein or proteins would be  
30 administered to a human host. If the organism is known to cause a disease in a nonhuman animal, then the vaccine including the active protein would be administered to a nonhuman animal.

In formulating a vaccine, the active protein  
35 is employed in the vaccine in an amount effective to provide protection against the disease caused by the organism against which protection is sought. In

general, each dose of the vaccine contains at least 5 micrograms and preferably at least 100 micrograms of the active protein. In most cases, the vaccine does not include the active protein in an amount greater than 20 milligrams.

The term "protection" or "protecting" when used with respect to a vaccine means that the vaccine prevents the disease or reduces the severity of the disease.

The active protein is employed in conjunction with a physiologically acceptable vehicle to provide protection against the organism. As representative examples of suitable vaccines in carriers, there may be mentioned: mineral oil, alum, synthetic polymers, etc. Vehicles for vaccines are well known in the art and the selection of a suitable vehicle is deemed to be within the scope of those skilled in the art from the teachings herein. The selection of a suitable vehicle is also dependent upon the manner in which the vaccine is to be administered. The vaccine may be in the form of an injectable dose and may be administered intra-muscularly, intravenously, or by sub-cutaneous administration. It is also possible to administer the vaccine orally by mixing the active components with feed or water; providing a tablet form, etc.

Other means for administering the vaccine should be apparent to those skilled in the art from the teachings herein; accordingly, the scope of the invention is not limited to a particular delivery form.

It is to be understood that a vaccine may also be formulated by use of an antibody elicited in response to a homologous protein of the organism.

The protein and/or antibody used in the vaccine is essentially free of the organism; i.e., cellular matter.

In accordance with another aspect of the present invention, there is provided a diagnostic kit and/or assay for determining an organism which employs in the assay and/or kit an antigen which is recognized  
5 by an antibody elicited by a protein of the organism which has at least 50% homology with a T. cruzi heat shock protein, as hereinabove described, i.e., a "homologous protein" of the organism.

The antigen employed as a diagnostic may be  
10 obtained or produced as hereinabove described with reference to the active protein included in the vaccine.

In accordance with yet a further aspect of the present invention, there is provided a diagnostic  
15 assay and/or reagent for determining an organism which includes and/or employs an antibody (or fragment thereof) which recognizes an antigen of the organism to be determined, which antigen of the organism has at least 50% homology with a heat shock protein of T.  
20 cruzi, as hereinabove described.

The antibody employed in the assay and/or assay kit may be either a polyclonal or monoclonal antibody elicited in response to a homologous protein. In particular, the antibody employed in the diagnostic  
25 assay and/or kit is elicited in response to a protein and/or fragment and/or derivative thereof having at least 50% homology with a heat shock protein of T. cruzi.

A diagnostic kit and/or assay for determining  
30 an organism which includes a homologous protein may be formulated to determine such organism by a variety of procedure.

For example, the organism may be determined by a so-called sandwich assay kit or assay for  
35 determining the organism by determining in a sample (derived from a host containing or suspected of

containing the organism) antibody elicited in response to a homologous protein of the organism. In this procedure, antigen of the type hereinabove described is contacted with the sample under conditions at which any of such antibody present in the sample is immunobound to the antigen, which antigen is preferably supported on a solid support.

Antibody bound to such antigen may then be determined by use of an appropriate tracer comprised of a ligand bound or recognized by such antibody labeled with a detectable marker or label. The ligand of the tracer may be, for example, an antibody bound by or recognized by the bound antibody.

The marker may be any one of a wide variety of labels (for example a radioactive label, an enzyme label, a chromogen label, etc.).

The techniques for forming such an assay and for providing a tracer are known in the art and no further details in this respect are deemed necessary for understanding the present invention.

For example, there may be employed a so-called ELISA sandwich assay format in which a plastic microtiter plate is coated with an antigen of the type described (one which is recognized by antibody elicited in response to homologous protein of the organism) and sample derived from a host suspected of containing the organism is incubated with the coated antigen. After appropriate washing, labeled immunoglobulin (antiglobulin to the host species which is suspected of containing the organism) labeled with a detectable enzyme (for example horseradish peroxidase or alkaline phosphatase) is incubated with the antibody bound by the coated antigen. After washing, an appropriate developer is added.

Alternatively, an agglutination assay may be employed in which case particles, such as polystyrene

beads, coated with the appropriate antigen is mixed with appropriate sample, and presence of antibody is detected by agglutination.

These and other procedures should be apparent to those skilled in the art.

In an alternative sandwich immunoassay format, an antibody of the type hereinabove described may be employed to directly determine a homologous heat shock antigen or protein of the organism to be determined. For example, a sample (derived from a host containing or suspected of containing the organism) is subjected to a sandwich assay by contacting the sample with an antibody (or fragment thereof) which recognizes the homologous heat shock antigen of the organism, which antibody is preferably supported on a solid support. Such contacting is effected under conditions which will immunobind the homologous heat shock antigen (if present) to the antibody. Thereafter, bound antigen may be determined by use of a tracer comprised of a ligand (which is bound by or recognizes the homologous antigen) labeled with a detectable marker or label. Thus, for example, the tracer may be labeled antibody elicited in response to the homologous antigen of the organism. As hereinabove indicated, the antibodies capable of recognizing a homologous protein of the organism may be a monoclonal and/or polyclonal antibody.

In this assay format, which employs an antibody which recognizes a homologous protein of the organism, markers (labels) and techniques, as hereinabove described and as known in the art, may also be employed.

The assay or reagent kit which employs antigen and/or antibody of the type hereinabove described may be included in an appropriate reagent kit package. The package may include other materials



useful in the assay, for example, tracer, buffers, standards, etc., in appropriate reagent containers.

In accordance with another aspect of the present invention, there is provided an assay and/or  
5 reagent kit for determining the presence of an organism which includes or employs a DNA probe which encodes for a protein of the organism having at least 50% homology with a heat shock protein of *T. cruzi* as hereinabove described.

10 The DNA probe which is used may be all or a portion of the DNA which encodes for a homologous protein. If a portion of the DNA which encodes for a homologous protein is employed, such DNA portion should include a portion of the DNA which encodes for  
15 a variable region of the homologous protein.

Accordingly, the DNA probe is employed under conditions whereby hybridization is accomplished over at least a portion of the DNA which encodes for a variable region (preferably a hypervariable region)  
20 of the homologous protein.

The hybridization may be performed with a suitably labeled form of the DNA (for example  $^{32}\text{P}$ , although other detectable labels, including non-radioactive labels may be used) in a procedure  
25 similar to the procedure for identifying DNA of the organism encoding for a protein having the requisite homology with a *T. cruzi* heat shock protein.

The present invention will be further described with respect to the following examples;  
30 however, the scope of the invention is not to be limited thereby. Unless otherwise indicated, all methods and abbreviations are well known in the art and are found in Maniatis. All references in this document are hereby incorporated by reference herein.

Example 1 -- Trypanosoma Cruzi Heat Shock Protein and Its Reaction with Sera from Infected Persons.

A. Growth and Isolation of Parasites

Trypanosoma cruzi, Peru strain, was used in all experiments. Epimastigotes were grown at 28°C in modified HM (Warren, S. Parasitology, 46:529-539, 1960); 37 g/l brain heart infusion (Difco Lab., Detroit, MI), 2.5 mg/l hemin, 10% heat-inactivated fetal calf serum. Log phase cells were harvested by centrifugation and washed twice with cold PSG (20 mM sodium phosphate, pH 7.4, 0.9% NaCl, 1.0% glucose). Culture form trypomastigotes were obtained from infected Va-13 cells as previously described. See Sanderson et al., Parasitology, 80:153-162, (1980), and Lanar and Manning, Mol. and Biochem., Parasitology, 11:119-131, (1984).

B. Isolation of DNA and RNA

Parasites were harvested from culture by centrifugation and washed several times with PSG (20 mM sodium phosphate, pH 7.4, 0.9% NaCl, 1.0% glucose). Epimastigotes were resuspended at a concentration of  $10^9$ /ml in PEG/EGTA buffer (20 mM Tris-HCl, pH 7.6, 25 mM EGTA, 50 mM MgCl, 25mM CaCl, 1.0% Triton-X100, and 4mM dithiothreitol), plus 250 u/ml of RNase in (Promega Biotec, Madison, WI), incubated on ice for 20 min., centrifuged at 8000 x g for 15 minutes at 4°C. The supernatant containing the RNA was phenol extracted 3 times, then extracted once with chloroformisoamyl alcohol (24:1) and ethanol precipitated. The pellet (nuclei and kinetoplasts) was resuspended at a concentration of  $10^9$  parasite equivalents/ml in 10 mM Tris-HCl, pH 8.0, 50 mM EDTA, 0.1% SDS, 150 ug/ml Proteinase K (Boehringer- Mannheim, Indianapolis, IN) and incubated at 65°C for 1 hour. After cooling to room temperature, the DNA was gently extracted with an equal volume of phenol for 1 hour. This extraction

was repeated once, and the aqueous phase was extracted with chloroform-isoamyl alcohol (24:1) once. The DNA was recovered by ethanol precipitation. The DNA pellet was gently redissolved in 10 mM

5 Tris-HCl, pH 8.0, 1 mM EDTA and treated with 0.15 mg/ml DNase-free RNase A for 30 minutes at room temperature. After RNase digestion the sample was extracted once with phenol, once with chloroformisoamyl alcohol, and then precipitated with ethanol. The size of the DNA was

10 determined to be greater than 20 kilobase pairs (kb) on agarose gels. Trypomastigote DNA and RNA was prepared in an identical manner except that the parasites were resuspended at a concentration of  $5 \times 10^9$ /ml.

#### C. Preparation of A+ mRNA

15 Poly A+ containing RNA was isolated by Oligo(dT)-cellulose chromatography (Aviv and Leder, J. Immunol., 127:855-859, 1972). Total RNA was loaded onto an oligo (dT)-cellulose column (Type 3, Collaborative Research, Lexington, MA) in 10 mM

20 Tris-HCl, pH 7.5, 1 mM EDTA, 0.2% SDS, 400 mM LiCl. RNA was eluted from the column at 40°C with 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.2% SDS.

#### D. Construction of the T. cruzi "Sau3a Partial" Genomic Library in Bacteriophage EMBL3

25 200 µg of T.cruzi epimastigote DNA was digested with the restriction endonuclease Sau3A (Boehringer-Mannheim, Indianapolis, IN) according to manufacturer's specifications. Aliquots of the reaction were removed at 1, 2.5, 5, 10, 20, 40 and 60

30 minutes. Upon removal each aliquot was diluted to 25 mM in EDTA and heated for 15 minutes at 68°C. The samples were pooled, the DNA was size fractionated over a Sephacryl S-1000 column (Pharmacia, Piscataway, NJ) in 200 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA.

35 Those fractions containing DNA in size from 5 kb to 20

kd were pooled, ethanol precipitated, and used for cloning. The lambda bacteriophage cloning vector EMBL3 (Frishauf et al., J. Mol. Biol., 170:827-842, 1983) was used. EMBL3 arms and GIGAPAK packaging system were  
5 purchased from Vector Cloning Systems (San Diego, CA) and used according to the manufacturer's instructions.

#### E. Hybridization-Selection/Translation

Specific *T. cruzi* RNAs were purified from total *T. cruzi* RNA using the technique of  
10 hybridization-selection/translation as described by Riccardi et al., PNAS, 76:4927-4931, 1972. 25-50 ug of purified plasmid DNA was digested with an appropriate restriction endonuclease (to linearize the plasmid), the DNA was cleaned by phenol extraction and chloroform  
15 extraction and denatured by boiling for 10 minutes. Following boiling, the DNA was quick-frozen, thawed, then spotted onto a 9mm diameter nitrocellulose filter. The filter was washed several times with 6XSSC, then air dried and baked for 2 hours at 80°C in vacuo. For  
20 hybridization, 100 µg of *T. cruzi* total RNA was reacted with the DNA containing filter in a solution containing 65% formamide, 0.01 M PIPES, pH 6.4, 0.4 M NaCl at 65°C for 3 hours. Following the hybridization reaction, the filter was washed 10 times with 1XSSC,  
25 0.1% SDS at 60°C, 3 times with 0.002 M EDTA at 60°C, and once with water at room temperature. The specifically hybridized mRNA is eluted from the filter by boiling the filter in a small volume of water for two minutes, quick-freezing the solution, then ethanol  
30 precipitating the RNA. The purified RNA is resuspended in water, then translated in an in vitro translation system (such as rabbit reticulocyte).

#### F. Immunoprecipitation Reactions

A 1:10 to 1:50 dilution of individual serum was  
35 prepared using the 10 mM Tris-HCl, pH7.5, 1% Nonidet

P-40 (NP 40), 1 mM N-alpha-p-tosyl-L-Lysine chloromethyl ketone (TLCK), 1 mM phenyl methyl sulfonyl fluoride (PMSF), and 2.8 Kallikrein Inactivator Units (KIU)/ml aprotinin. The diluted  
5 serum was mixed with an equal volume of cell-free translation reaction mixture, and incubated overnight at 4°C. 10 µl of 10% protein-A-Sepharose (Pharmacia, Piscataway, NJ) was added and gently mixed for 1 hour at 4°C. The immune complexes were washed and analyzed  
10 on SDS-polyacrylamide gels as described in Dragon et al., Mol. and Biochem. Parasitology, 16:213-229, 1985.

#### G. Synthesis of cDNA

cDNA was synthesized by methods known to those of ordinary skill in the art. Briefly, 2 µg of  
15 epimastigote or trypomastigote A+ mRNA was transcribed by the action of AMV reverse transcriptase as described by Ullrich et al., Science, 196:1313-1319, (1977) and Gubler, Gene, 25:263-269, (1983). Transcription was initiated at the 3' polyadenylated end of the mRNA  
20 using oligo(dt) as a primer. The second strand was copied using DNA polymerase I and RNase H (Boehringer-Mannheim, Indianapolis, IN) and appropriate buffers.

Specifically, 2 µg of oligo-dT (12-18  
25 nucleotides, Pharmacia Molecular Biology Division, Piscataway, NJ) was annealed to 2 micrograms of purified mRNA in the presence of 50 mM NaCl. The annealing reaction was heated to 90°C and then slowly cooled. For the reverse transcriptase reaction,  
30 deoxynucleosidetriphosphates (dATP, dTTP, dGTP and dCTP) were added to make a final concentration of 0.5 mM, along with 40 units of enzyme (Molecular Genetic Resources, Tampa, FL). The reverse transcriptase reaction buffer contained 15 mM Tris-HCl, pH 8.3, 21  
35 mM KCl, 8 mM MgCl<sub>2</sub>, 0.1 mM EDTA. and 30 mM beta-mercaptoethanol. This mixture was incubated at 42°C

for 45 minutes. The RNA-DNA duplex was extracted once with phenol chloroform and then precipitated with ethanol. The pelleted material was then resuspended in 100 microliter reaction mixture containing the  
5 following: 20 mM Tris-HCl pH 7.5, 5 mM MgCl<sub>2</sub>, 100 mM KCl and 250 uM each dATP, dCTP, dTTP, dGTP.

RNAase H (100 units/ml Pharmacia Molecular Biology Division, Piscataway, NJ) and DNA Polymerase I -- Klenow fragment (50 units/ml Boehringer  
10 Mannheim, Indianapolis, IN) were added and the reaction was incubated at 12°C for 60 minutes. The combined activities of these enzymes result in the displacement of the mRNA from the RNA-DNA duplex as the first cDNA strand is used as a template for  
15 synthesis of the second cDNA strand. The reaction was stopped by the addition of EDTA to a final concentration of 10 mM and the DNA duplex was then extracted with phenol: chloroform and ethanol precipitated. The sequence of the reactions of DNA  
20 Polymerase I and RNAase H was predicted to yield cDNA molecules which were blunt ended at both their 3' and 5' ends. A 3' blunt end is necessary for the subsequent cloning of the cDNA.

#### H. Construction of the cDNA Library

25 Briefly, the double stranded cDNA preparations were digested with the restriction endonucleases SacI and PvuII (New England Biolabs, Beverly, MA) and ligated, using T4 DNA ligase, into the SacI and SmaI sites of the plasmid pUC18 (Yanish-Perron  
30 et al., Gene, 33:103-119, 1985). This mixture was used to transform E. coli K12 strain JM83, selecting for ampicillin resistance conferred by the introduction of the pUC18 into the host cell. From 2 ug of mRNA approximately 150 ng of cDNA were prepared which  
35 yielded about 7000 ampicillin resistant clones.

More specifically, the cDNA was resuspended in 100 microliters of sterile water. Approximately 50 ng was digested with SacI (5000 units/ml) and pVUII (12000 units/ml) in the presence of 6 mM Tris-HCl (pH 7.4) 6 mM MgCl<sub>2</sub> and 6 mM beta-mercaptoethanol for 60 minutes at 37°C.

The sample was then re-extracted with phenol: chloroform and ethanol precipitated. For the cloning step a pUC18 vector was used. The vector had been digested with SacI and SmaI. SmaI provided the blunt end site necessary for ligation of the 3' end of the cDNA. The ligation reaction was performed using 40 ng of vector DNA and 50 ng of cDNA. Ligation was done overnight at 12°C in a ligase buffer of 50 mM Tris-HCl (pH 7.8), 10 mM MgCl<sub>2</sub>, 20 mM dithiothreitol, 1.0 mM rATP using one unit of T4 DNA ligase.

The recombinant DNA molecules were then introduced into E. coli K-12 strain JM83 by transformation. The transformed bacteria were spread on agar plates containing the antibiotic ampicillin at a concentration of 50 micrograms/ml. Since the plasmid pUC18 contains the ampicillin resistance gene, only those bacteria which acquired a recombinant plasmid survived. These bacteria each grew and divided to form a bacterial colony. Each cell in the colony is a descendant of the original parental cell and contains the same recombinant plasmid. Using hybridization - selection/translation and immunoprecipitation techniques to screen the cDNA library a clone was identified which contained nucleotide sequences corresponding to a 70 kd T. cruzi peptide.

#### I. Isolation of the full length 70 kd gene

The cDNA clone was used as a probe to screen the T. cruzi Sau3a partial genomic library as described by Maniatis et al. A lambda phage designated FG21 was identified which contained multiple copies of the 70 kd

gene. A 2.4 kb SmaI fragment was sub-cloned into pUC9 from FG 21. This subclone called pEG22 contained one full length copy of the 70 kD gene. The DNA sequence of PEG22 was determined. FG21, was sequenced and used to construct an expression plasmid to allow production of the 70 kd antigen in E. coli.

#### J. Expression of Cloned Genes in E. coli

Several systems are available in the laboratory for expressions of foreign genes in E. coli and other mammalian and bacterial tissue culture cell lines. It is important to provide the cloned genes with an E. coli ribosome binding site for initiation of translation and a strong promotor to obtain sufficiently high levels of protein. Although obtaining "direct" expression of the protein is possible, it appears to be more efficient to produce the protein as a fusion protein, the amino terminus of which is a small part of an E. coli protein containing signals for the initiation of protein synthesis. The amino terminus of B-lactamase and the amino terminus of B-galactosidase can make such fusion proteins [Hegpeth et al., Mol. Genet., 163:197-203 (1980) and Lingappa et al., PNAS, 81:456-460 (1984)]. These and other systems may be used to obtain expression of the cloned gene.

Sequencing analysis showed that the coding region of the 70 kd gene was flanked by an AhaIII site 30 base pairs upstream from the putative ATG start codon. An additional AhaIII site is located 367 base pairs following the TGA stop codon in the nucleotide sequence of FG21. Subsequently FG21 was digested with the restriction enzyme AhaIII. The resulting DNA fragment was 2,341 base pairs long. It was gel purified and cloned in the SmaI site of the expression vector pUC9. The resulting plasmid, pFP70-47, was used to transform E. coli K12 SG936 bacteria.



A sample of this recombinant bacteria has been placed on deposit with the American Type Culture Collection (12301 Parklawn Drive, Rockville, Maryland, USA) as ATCC number 67254. The culture was deposited  
5 on November 4, 1986. This strain, SG936/FP70-47 produces a 70 kd polypeptide which can react with chagasic sera. Expression of the entire protein, however, provides as many determinants as possible on the target antigen.

#### 10 K. Antigen Production

The transformed E. coli are grown in liquid culture containing 50 micrograms per ml of ampicillin to enhance plasmid ability. Cultures are harvested at an OD of 2.0 measured at 550 nm. The cells are then  
15 pelleted and washed and lysed by freeze/thaw and sonication. A detergent extraction solubilizes most of the remaining polypeptides. The 70 kd expressed product, however, remains insoluble and is harvested by centrifugation. This insoluble "cement" is denatured  
20 in urea and subsequently diluted at a high pH and the pH is then adjusted back to neutral. During the renaturation process the antigen refolds and achieves that immunologically active conformation. The details of this procedure used are identical to those used to  
25 restore enzyme activity to recombinant chymosin as described by McCaman et al., J. Biotech., 12:117-191, (1985).

#### Example 2 -- 74.5 kda M. Hyo Antigen and Use As a Vaccine

##### 30 A. Preparation of M. hyopneumoniae DNA

Strain P-57223 (obtained from Dr. Charles Armstrong, Purdue University) was grown in 1 liter of Friis medium to a density of approximately  $10^9$  to  $10^{10}$  color changing units per ml. The cells were  
35 harvested by centrifugation and resuspended in 2 ml

phosphate buffered saline which brought the total volume to 3.25 ml. The suspension was then mixed with a solution consisting of 24.53 g cesium chloride dissolved in 19.75 ml 10 mM Tris pH 8.0 1 mM EDTA and 1.53 of 10 mg/ml ethidium bromide was added. This was mixed with a solution consisting of 3.87 g cesium chloride dissolved in 2.15 ml 10 mM Tris pH 8.0. 1 mM EDTA, 8.9% Sarkosyl. The resulting suspension was incubated at 65°C for 10 minutes to completely lyse the cells. The DNA was separated by equilibrium buoyant density centrifugation in a Sorvall TV850 rotor at 43,000 rpm for 18 hours, and withdrawn with an 18 gauge needle. This DNA was subjected to two additional buoyant density centrifugations in a Sorvall TV865 rotor at 55,000 rpm for 7 and 18 hours respectively, each time the band of genomic DNA being removed with an 18 gauge needle. The resulting DNA solution was extracted with cesium chloride saturated isopropanol, to remove ethidium bromide, and extensively dialyzed against 10 mM Tris pH 8.0, 1mM EDTA, to remove the isopropanol and cesium chloride.

#### B. DNA Probing of M. hyopneumonia DNA

Plasmid pEG22, described in Example 1 is purified from E. coli by methods in the art, and labeled with <sup>32</sup>p by nick translation using DNA polymerase I.

pEG22 is used as a probe as follows:

Mycoplasma genomic DNA was digested with EcoRI under the following conditions at 37°C for 2 hours.

114 microliters P-5722-3 DNA

6 microliters H<sub>2</sub>O

15 microliters 10X BRL-3 (Bethesda Research Labs)

15 microliters EcoRI (Bethesda Research Labs)

67 microliters were mixed with 0.1% Bromphenol blue, glycerol, loaded onto a 1% agarose gel and electrophoresed until the blue color had migrated to within 1cm of gel end. The DNA was transferred to a nitrocellulose filter by Southern's technique. The filter was hybridized to the DNA probe described above under conditions which allow hybridization in the absence of exact sequence identity.

Hybridization:

10                   6 X NET  
                    5 x Denhardts solution  
                    2 X  $10^6$  counts per minute probe,  
                    37°C for 18 hours

Wash:

15                   6 X NET  
                    0.1% SDS  
                    3 times at room temperature,  
                    1 time at 50°C

                    6 X NET  
20                   1 M NaCl  
                    90 mM Tris pH 7.6  
                    6 mM EDTA

Southern blot analysis shows that the DNA probe hybridized to a specific EcoRI restriction endonuclease fragment of approximately 6 kB in length and thus include the antigen's gene.

C. Cloning the Gene by Hybridization

In order to identify the gene by hybridization to the pEG22 DNA probe, 200 micrograms of P-57223 DNA was digested with 120 units of EcoRI in a volume of 600 microliters. The digestion mixture was mixed with glycerol and xylene cyanol blue FF and electrophoresed on a 3.25% acrylamide gel. Five

slices of approximately 0.5 cm were cut from the gel in the size range desired and electroeluted in 0.1% SDS, 0.5 X TBE buffer. The resulting DNA fractions were extracted with phenol/chloroform, ethanol  
5 precipitated, and each resuspended in 50 microliters of 10mM Tris pH 8.0, 1mM EDTA. By dot-blot analysis, (See Nuc. Acid Res. 7:1541-1552, 1979), fraction 4 was shown to contain the DNA fragment of interest.

To create a gene library enriched for the  
10 desired fragment, 7 microliters of Fraction 4 was ligated to EcoRI digested pUC9 with T4 ligase one-half of the reaction was transformed into JM83 and plated on X-gal plates where white colonies contain plasmids and inserts. Plasmid DNA from 24 white colonies was  
15 prepared and transferred to nitrocellulose by the slot-blot modification of the dot-blot procedure and probed with <sup>32</sup>P labeled pEG22.

Plasmid DNA preparations which hybridize to the DNA probe are subjected to EcoRI digest analysis to  
20 show that each plasmid contains the same size insert fragment, and most likely the same gene. A plasmid is selected for DNA sequence analysis which shows greater than 50% identity to pEG22.

#### D. Preparation of Genomic Library

25 A preparative digest of 200 µg genomic DNA of Mycoplasma hyopneumoniae P-57223 was done using 200 units of EcoRI in a total volume of 1 ml and 250 µl aliquots were removed at 6 min, 25 min, 42 min and 63 min.

30 The four preparative samples of partially digested Mycoplasma DNA were then combined (200 µg) and loaded onto an exponential sucrose gradient. The gradient was centrifuged in a Sorvall AH627 rotor at 26 k rpm for 21 hrs at 15°C.

35 The gradient was then slowly fractionated from the bottom by collecting 15 drop fractions (90

fractions total). 20  $\mu$ l of each fraction was then run on a 1% agarose gel as described above. Fractions containing DNA fragments smaller than 18 kbp and larger than 15 kbp were pooled (fractions 32-40) and dialyzed against TE (10 mM Tris.HCl pH 7.5, 1 mM EDTA pH 8.0) to remove the sucrose. The DNA (3.5ml) was then precipitated with ethanol and resuspended to about 15  $\mu$ l (1 mg/ml) under vacuum and stored at -20°C.

EcoRI Arms of bacteriophage lambda-Dash were obtained from Vector Cloning Systems (StrataGene) and were ligated at a concentration of 200  $\mu$ g/ml to Mycoplasma target DNA at a concentration of 25  $\mu$ g/ml in a total volume of 10  $\mu$ l using T4 ligase (Boehringer GmbH) at a concentration of 100 units/ml. The ligation reaction was incubated at room temperature for 2 hours. 4  $\mu$ l of the ligation was then packaged into lambda particles using the in vitro packaging kit Gigapack (StrataGene). The phage was then titered on E. coli strain P2392 (StrataGene) and found to be  $7.75 \times 10^5$  pfu/ml ( $3.1 \times 10^5$  pfu/ $\mu$ g of lambda-Dash).

#### E. Screening of Library

The library is screened using the plasmid previously obtained which shows greater than 50% homology to pEG22, by the previously described probing procedure. DNA from positive recombinants is prepared, digested with EcoRI, analyzed by gel electrophoresis, to indicate portions of the M. hyopneumoniae genome composed of several EcoRI restriction fragments. One of the fragments is digested with EcoRI, ligated to EcoRI digested PWA148 and transformed into E. coli strain JM83 and called pMYCO16; its DNA was prepared and digested with a number of different restriction endonucleases in order to derive the restriction map shown in Figure 3.

Plasmid pWHA148 is prepared by inserting a synthetic oligonucleotide into the Hind III site of pUC18. The amino terminal coding sequence of the X-complementing peptide of B-galactosidase is shown in Figure 4, and contains 8 additional restriction sites over the parent pUC18. The oligonucleotide insert into pUC18 is shown in Figure 4 between the SphI and Hind III sites.

An N-terminal portion of pEG22 is used by Southern analysis to hybridize to the 0.6kb AccI-AsuII restriction fragment of pMYC016. DNA sequence analysis of the 0.6 kb fragment identifies that start codon of the homologous gene.

On the restriction map of pMYC016 (Figure 3) the gene begins within the 0.6 kb AccI-AsuII restriction fragment, extends clockwise within the 0.4 kb AsuII - ClaI, 1.2 kb ClaI - ClaI, and 1.4 kb ClaI-HindIII fragments, and ends short of the HindIII site. DNA sequence analysis shows that pMYC016 contains a 74.5 kD protein homologous to the 70 kD T. cruzi heat shock antigen.

The DNA-amino acid sequence of the 74.5 kD gene is shown in Figure 5.

#### F. Expression of full length M.hyo. 74.5 kD antigen in E. Coli

Plasmid pMYC016 DNA (Figure 3) was digested with AccI, treated with Mung Bean nuclease to remove the single stranded AccI tails, re-ligated to delete the 1.9 kb AccI fragment in front of the 74.5 kD antigen gene and transformed into E. coli strain JM83. One transformant was named pMYC029; its DNA was digested with a number of different restriction endonucleases in order to derive the restriction map shown in Figure 6.

pMYC029 was subjected to DNA sequence analysis which showed that a spontaneous deletion had occurred at the ligation juncture, where two bases were deleted and the PstI site was retained, as shown  
5 below (only a portion of the 5' to 3' strands are represented).

pMYC029 expected: TTGCATGCCTGCAGGTACTTTCTTTTGTCT

PstI

pMYC029 observed: TTGCATGCCTGCAGGCTTTCTTTTGTCT

10

PstI

This fortuitous deletion allows the in frame insertion into the pUC9 open reading frame. Plasmid pMYC029 is a low level expression plasmid.

15 G. Construction of pMYC031 and expression of 74.5 kD antigen fragment

Because the mycoplasma insert of pMYC029 is oriented away from the Lac promoter of pWHA148, it was desired to insert the gene into another expression vector, pUC9. The two base deletion  
20 enabled the gene for the 74.5 kD antigen to be placed in the same reading frame as the beta-galactosidase gene of E. coli vector pUC9.

In order to perform this construction, pMYC029 DNA was digested with PstI and EcoRI, the PstI  
25 - EcoRI fragment containing the entire 74.5 kD coding sequence was purified, ligated to the PstI and EcoRI digested vector pUC9, and transformed into E. coli strain JM83. One transformant was named pMYC031 (Figure 7); its DNA was prepared and transformed into  
30 E. coli strain W3110 by the transformation procedure described above.

#### H. Construction of pMYC032

It is known that TGA codons encode the amino acid tryptophan in mycoplasma but normally terminate peptide chain elongation in *E. coli* and that the  
5 trpT176 gene, a mutant tryptophan t-RNA which recognizes UGA (Raftery, et al., Jour. Bacteriol., 158:849-859), allows peptide chain elongation at TGA codons in *E. coli* laboratory mutants. We reasoned that the addition of trpT176 to expression vectors would  
10 allow *E. coli* peptide chain elongation at the mycoplasma TGA codons of cloned genes.

Plasmid pCAM101 was purchased from James Curran (University of Colorado) as a convenient source of the trpT176 gene and is shown in Figure 8.

15 DNA from pCAM101 was digested with EcoRI, the 0.3 kb EcoRI fragment which contains the trpT176 gene was purified, ligated to EcoRI digested pMYC031, and transformed into *E. coli* strain W3110. One transformant was named pMYC032 and its restriction  
20 map is shown in Figure 9.

#### I. Expression of *M. hyopneumoniae* 74.5 kD antigen in *E. coli*

A W3110 (pMYC032) transformant was selected, grown in L-broth, lysated as previously described, and  
25 a portion subjected to polyacrylamide gel electrophoresis. New 75 kD and 43 kD proteins were identified by gel electrophoresis which represented approximately 5% and 0.1% of total *E. coli* protein, respectively. The pMYC032 75 kD  
30 protein was shown by Western blot to react with the previously described pig antisera raised against the 74.5 kD *M. hyopneumoniae* antigen.

An improved expression plasmid pMYC087 has been deposited with the ATCC on June 30, 1989 as ATCC  
35 number 68030. It contains an in vitro change of TGA to TGG (Tryptophane) at codon position 211 (see Figure 5).



J. Use of the recombinant form of Mycoplasma hyopneumoniae 74.5 kD antigen as a vaccine

A W3110 (pMYC032) transformant from Example 2 was selected, grown in M-9 minimal medium in a 14 liter Chemap fermenter to a cell density of 110 O.D. 600, and 120 g (wet weight) of cells were harvested from 500 ml by centrifugation. A suspension was prepared consisting of 2.3 g of cells per 10 ml of PBS containing 12 mM EDTA, 0.5 mg/ml lysozyme. The suspension was incubated at 25 °C for 15 minutes. sonicated on ice for 2 minutes in 30 second bursts, centrifuged at 13,000 g for 10 minutes at 4°C, and the soluble fraction reserved as product. A portion of the product was subjected to polyacrylamide gel electrophoresis. The recombinant form of 74.5 kD antigen made up approximately 25% of the soluble protein and the yield dosages were prepared in PBS at 100 and 500 µg per dose and emulsified on ice with equal volumes of Freund's incomplete adjuvant (Sigma) immediately prior to use.

Vaccination Test

- Week 0 Three litters of Hampshire, Hampshire X Duroc, and York piglets taken by Caesarian section.
- 25 Week 1 Piglets divided randomly into 7 pig dosage groups and each vaccinated sub-cutaneously in leg.
- Week 3 Booster vaccination, as above, opposite leg.
- 30 Week 8 Challenge administered by trans-tracheal inoculation of  $10^6$  CCU Mycoplasma hyopneumoniae.
- Week 12 Necropsy of experimental animals and infection controls.

The results were as follows:

<u>Group</u>	<u>Incidence*</u>	<u>Severity**</u>
Control	5/5	12.4 $\pm$ 4.7
100 ug 74.5 kD	1/4	4.2 $\pm$ 4.9
5 100 ug recomb. 74.5 kD	2/6	9.7 $\pm$ 11.7
500 ug recomb. 74.5 kD	4/4	25.0 $\pm$ 6.1

\* Number of pigs with a lung lesion score greater than 5%

10 \*\* % of lung surface effected (mean  $\pm$  std. dev.)

Example 3. -- The 70 kD Hsp Analog from Mycoplasma Gallisepticum.

#### A. Preparation of Genomic Libraries

15 Two strains of M. gallisepticum F-K810 and R, were obtained from R. Yamamoto (U. C. Davis) and grown in F-80 media for the preparation of genomic DNA. (Nord Veterinaarmed. 27:337-339).

20 Approximately 22 ml of stationary phase M. gallisepticum culture was centrifuged at 13,000 X g at 4°C for 10 minutes to harvest mycoplasma cells. The supernatant was discarded and the cell pellet was resuspended in PBS to wash. Cells were harvested by centrifugation after washing. The cells were washed a total of three times with PBS and the resulting cell  
25 pellet frozen at -78°C. After thawing, the cells were resuspended in 2 ml 10 mM Tris-HCl pH 8.0, 50 mM EDTA, 1% SDS, and 100  $\mu$ g Proteinase K was added. The cells were lysed at 50°C for one hour with occasional mixing. The lysate was extracted with phenol then with  
30 chloroform/isoamyl alcohol to remove cellular debris. The DNA-containing aqueous phase was dialyzed against 4 liters of 10 mM Tris-HCl, 5 mM EDTA twice, and 10 mM Tris-HCl, 1 mM EDTA once. From each strain, 60  $\mu$ g of DNA was recovered, an amount sufficient for restriction  
35 analyses. Southern blot analyses, and library construction. Restriction digests indicated that the

two strains are similar to each other with limited restriction fragment length polymorphism.

B. Mixed oligonucleotide probes for isolating the Hsp70 protein from M. gallisepticum

5                   When the Hsp70 amino acid sequence from T. Cruzi aligned with the amino acid sequence of the M. hyopneumoniae 74.5 kD antigen of Example 2. Several regions containing sequences six amino acids in length are identical in both sequences. The array of DNA  
10 sequences which could encode these amino acid regions was determined. The two amino acid sequences corresponding to nucleotide sequences having the lowest degeneracy, were selected for use as oligonucleotide probes. These were synthesized as follows:

15    COD1159    Ile-Ile-Asn-Glu-Pro-Thr  
                  ATA-ATA-AAC-GAA-CCA-AC  
                  C    C    T    G    C  
                  T    T                G  
  T

20    COD1218    Gly-Gly-Gly-Thr-Phe-Asp  
                  GGA-GGA-GGA-ACA-TTC-GA  
                  C    C    C    C    T  
                  G    G    G    G  
                  T    T    T    T

25                   Pools of the above oligonucleotides were labeled with <sup>32</sup>P using polynucleotide kinase (BRL) and used to probe Southern transfers of HindIII digested M. gallisepticum chromosomal DNA. After 50°C washes in 6X NET, 0.1 SDS, COD 1159 hybridized to two HindIII  
30 fragments. COD 1218 hybridized to two HindIII fragments at 45°C under likewise identical conditions. Both probes hybridize to an apparently identical 3.4 kb fragment, where as the other fragments differ in length

and probably represent hybridization due to non-specific sequence homology. The hybridization of both probes to the same 3.4 kb HindIII fragment is highly significant as the probability that hybridization of both probes to the same fragment of genomic DNA results from non-specific sequence homology is less than  $2 \times 10^{-3}$ . The hybridization patterns for DNA purified from strain R strain and F-K810 strain of *M. gallisepticum* were identical to one another.

Plasmid DNA from pMYC087, containing the gene for *M. hyopneumoniae* (ATCC 68030 deposited with the American Type Culture Collection on June 30, 1989) was labeled using the Boehringer Mannheim nonradioactive Southern hybridization kit (Genius kit) and used to probe a Southern transfer of EcoRI and HindIII restriction digested chromosomal DNA from the F-strain and *M. hyopneumoniae* as a positive control. The probe detected bands of the expected size in the *M. hyopneumoniae* genome and an EcoRI band of 6.8 kb and a Hind III band of 3.3kb in the *M. gallisepticum* digests after washes at 65°C in 0.5X SSC and 0.1% SDS.

#### C. Preparation of Size Selected Genomic Libraries

The general approach for cloning the hsp antigen gene from *M. gallisepticum* was analogous to the procedure used for the *T. cruzi* 70 kD hsp. *M. gallisepticum* genomic DNA, 1 µg from both the R strain and the F-K8 I 0 strain, was digested to completion with the bacterial restriction endonuclease HindIII and separated on 3.25% polyacrylamide gels. DNA from four gel slices containing restriction digest fragments between 2 and 5 kb was electroeluted. An aliquot of DNA electroeluted from each of the four gel slices was subjected to agarose gel electrophoresis, transferred to a nitrocellulose membrane by Southern transfer and probed with <sup>32</sup>P-labeled COD1159 to identify the fraction which contains the 3.3kb hybridizing HindIII band. In

this way, a positive DNA fraction was identified. This positive DNA fraction was then ligated into Hind III digested pUC9 and transformed into E. coli DH5a.

D. Identification of Positive Clones

5 For each strain, 12 and F-K810, plasmid DNA from forty-eight recombinant clones was isolated by the method of Holms and Quigley 1981 (Anal. Biochem. 114:193-197, 1981), transferred to nitrocellulose using a Bio-Rad dot blot apparatus, and probed with COD1159  
10 in the case of the R-strain or both COD1159 and COD1218 on duplicate blots. In the case of strain F-K810.

One positive isolate was found for each strain. Plasmid pMGA4 contains a positive R-strain insert and has been deposited with the American Type  
15 Culture Collection on \_\_\_\_\_ 1989 with the designation \_\_\_\_\_. A map of pMGA4 is provided in Figure 10. The sequence of the M. gallisepticum Hsp70 DNA and the derived amino acid sequence is provided in Figure 11.

20 E. Expression, Purification and Use as a Vaccine

DNA from pCAM101 was digested with EcoRI, a 0.3 kb EcoRI fragment including trpT176 was purified, ligated to EcoRI digested pUC9, transformed into E. coli strain JM83, and one transformant was named  
25 pWHA160 (see Figure 12).

Plasmid pMGA4 DNA was digested with HindIII and BglIII, ligated to HindIII and BamHI digested pWHA160, digested with BamHI and BglIII, and transformed into E. coli strain DH5a. One transformant was named  
30 pMGA10. The MGA10 transformant was grown in L-broth at 37°C, and the cells harvested by centrifugation and frozen. The cell pellet from 4 ml of culture was resuspended in 100 µl of a solution consisting of 0.5 mg/ml hen egg-white lysozyme dissolved in 25 mM Tris pH  
35 8.0 10 mM EDTA; and incubated at 25°C for 10 minutes.

A portion of the resulting lysate was subjected to polyacrylamide gel electrophoresis and a new 67 kD protein was identified. Western blot analysis, using pig anti-74.5kD serum, showed that the new 67 kD  
5 protein was immunologically related to Hsp70.

F. Use of Bacterially Produced M. gallisepticum Hsp 70 Protein to Raise an Immune Response in Chicken

The purified M. gallisepticum protein is concentrated by lyophilization and resuspended to a  
10 final concentration of 0.5-2.0 mg/ml in 0.1% SDS. For use, the immunizing antigen is formulated in one volume of protein concentrate to three volumes of oil carrier consisting of 5% Arlacel, 94% Drakeol 6-VR and 1% Tween 80. The dose of the antigen employed is 100 µg/dose.  
15 Chicken receive the formulated vaccine by subcutaneous injection. A booster vaccination by the same route is done two weeks later.

Numerous modifications and variations of the present invention are possible in light of the above  
20 teachings; therefore, within the scope of the appended claims the invention may be practiced otherwise than as particularly described.

Table 1. Representative Pathogenic Organisms.

## 1: DISEASE AGENTS

## 1.1: BACTERIA

## 1.1.1: ACTINOBACILLUS SPP.

## 1.1.1.1: Actinobacillus lingiresii

Mastitis infections in cattle, sheep,  
swine, equine

1.1.1.2: Also known as Haemophilus  
swine pneumonia

## 1.1.2: BACILLUS SPP.

## Bacillus anthracis

Anthrax, an acute febrile disease of  
all mammals

## 1.1.3: BORDETELLA SPP.

1.1.3.1: B. bronchiseptica - respiratory disease in  
many species

## 1.1.3.2: B. pertussis - whooping cough in man

## 1.1.4: BORRELIA SPP.

1.1.4.1: B. burgdorferi - Lyme disease in dogs,  
deer, man

## 1.1.5: BRUCELLA SPP.

1.1.5.1: Brucella abortus, B. suis, B. melitensis  
brucellosis in cattle, sheep, swine,  
equine, canine, man

## 1.1.6: CAMPYLOBACTER SPP.

1.1.6.1: Campylobacter fetus  
causes infertility and embryonic  
death in cattle, swine, sheep,  
equine  
(vibriosis)

## 1.1.6.2: Vibrio cholerae - cholera in man

## 1.1.7: CHLAMYDIA SPP.

1.1.7.1: C. psittaci - respiratory disease in  
birds

## 1.1.7.2: C. cati - conjunctivitis in cats

## 1.1.8: CLOSTRIDIUM SPP.

1.1.8.1.: C. chauvoei  
blackleg in cattle and sheep1.1.8.2: C. septicum  
malignant edema in cattle and sheep1.1.8.3: C. haemolyticum  
red water in cattle1.1.8.4: C. novyi  
black disease in cattle and sheep1.1.8.5: C. sordelli  
big head disease in cattle and sheep1.1.8.6: C. perfringens  
enterotoxemia in cattle, sheep, swine,  
equine, gas gangrene in man

- 1.1.8.7: C. tetani  
tetanus in all mammals
- 1.1.8.8: C. botulinum  
8 types, causing botulism in all species
- 1.1.9: CORYNEBACTERIUM SPP.
  - 1.1.9.1: C. diphtheria - Diphtheria in man
  - 1.1.9.2: C. pyogenes - causes pyogenic processes in cattle, sheep, swine, goats
  - 1.1.9.3: C. renale - cystitis in cattle
  - 1.1.9.4: C. equi - pneumonia in horses
- 1.1.10.1: ERYSIPELOTHRIX SPP.
  - 1.1.10.1: Erysipelothrix rhusiopathiae - erysipelas in swine and man
- 1.1.11: HAEMOPHILUS SPP.
  - 1.1.11.1: H. influenza, respiratory disease in various species
  - 1.1.11.2: H. parainfluenza, H. parasuis, H. suis - respiratory disease in swine
- 1.1.12: KLEBSIELLA SPP.
  - 1.1.12.1: Klebsiella pneumoniae - Pneumonia and septicemia in animals and man
- 1.1.13: LISTERIA SPP.
  - 1.1.13.1: L. monocytogenes - Listeriosis -- encephalitis in ruminants
- 1.1.14: MYCOBACTERIUM SPP.
  - 1.1.14.1: M. tuberculosis, M. bovis, M. avium - Tuberculosis in various species
  - 1.1.14.2: M. paratuberculosis - Johne's disease in cattle, sheep, and goats
- 1.1.15: PASTEURELLA SPP.
  - 1.1.15.1: P. pestis - Plague in man and rodents
  - 1.1.15.2: P. haemolytica, P. multocida - respiratory disease in many species
- 1.1.16: PSEUDOMONAS SPP.
  - 1.1.16.1: P. aeruginosa - respiratory disease in various animals
  - 1.1.16.2: P. mallei - Glanders disease in dogs and cats
- 1.1.17: SALMONELLA SPP.
  - 1.1.17.1: S. typhimurium - enteric disease in a number of species
  - 1.1.17.2: S. typhisuis, S. choleraesuis - enteric disease in swine
  - 1.1.17.3: S. typhi - Typhoid fever



- 1.1.17.4: S. paratyphi - Paratyphoid - A in man
- 1.1.17.5: S. gallinarum - fowl typhoid
- 1.1.17.6: S. pullorum - pullorum disease in chickens

#### 1.1.18: STREPTOCOCCUS SPP.

- 1.1.18.1: S. agalactiae, S. dysgalactiae - mastitis in numerous species
- 1.1.18.2: S. dispar - enteritis in numerous species
- 1.1.18.3: S. equi - cholic in horses
- 1.1.18.4: S. genitalium - uterine infections in horses
- 1.1.18.5: S. pneumoniae - respiratory disease in man

#### 1.1.19: STAPHYLOCCUS SPP.

- 1.1.19.1: S. aureus - mastitis in many species
- 1.1.19.2: S. epidermidis - pyoderma in many species

#### 1.1.20: TULAREMIA SPP.

- 1.1.20.1: Francisella tularensis - Tularemia in man

#### 1.2.6: HERPESVIRIDAE

- 1.2.6.1: H. simplex Type 1 - Oral Herpes in man
- 1.2.6.2: H. simplex Type 2 - Genital Herpes in man
- 1.2.6.3: Epstein-Barr Virus - Mononucleosis in man
- 1.2.6.4: H. smiae - Herpes B. in primates
- 1.2.6.5: H. suis-Adjuskie's disease - pseudorabies in swine and cattle
- 1.2.6.6.: H. canis - Respiratory infection of dogs
- 1.2.6.7: H. equi - Equine rhinopneumonitis - respiratory and abortion in horses
- 1.2.6.8: H. bovis - IBR (Infectious Bovine Rhinotracheitis) in cattle
- 1.2.6.9: H. felis - FVR (Feline Viral Rhinotracheitis)
- 1.2.6.10: Laryngotracheitis virus - Laryngotracheitis in birds
- 1.2.6.11: Marek's Disease Virus - Marek's disease in birds
- 1.2.6.12: Feline calicivirus (FCV)
- 1.2.6.12: Cytomegaloviruses-many diseases in various animals

#### 1.2.13: POXVIRIDAE

- 1.2.13.1: SMALLPOX - WAS A MAJOR DISEASE IN MAN
- 1.2.13.2: VACCINIA - USED TO VACCINATE AGAINST SMALLPOX
- 1.2.13.3: COWPOX - SKIN DISEASE OF CATTLE
- 1.2.13.4: SWINEPOX - SKIN DISEASE OF SWINE
- 1.2.13.5: ECTROMELIA - MOUSEPOX
- 1.2.13.6: AVIPOXVIRUSES - FOWLPOX, CANARYPOX, PIEGEONPOX, TURKEYPOX,

- 1.2.13.7: CAPRIPOXIVIRUSES - LUMPY SKIN DISEASE IN SHEEP AND GOATS
- 1:2:13:8: PARAPOXIVIRUSES - "SORE MOUTH" IN SHEEP AND GOATS, BOVINE PAPULAR STOMATITIS

### 1.3: MYCOPLASMA

- 1.3.1: M. mycoides - Bovine respiratory disease
- 1.3.2: M. bovis - bovine mastitis
- 1.3.3: M. bovis genitalium - bovine epididymitis
- 1.3.4: M. bovoculi - Infectious bovine keratoconjunctivitis
- 1.3.5: M. bovirhinis and M. dispar - respiratory disease
- 1.3.6: M. hyorhinis and M. hyosynoviae - respiratory disease and lameness in swine
- 1.3.7: M. gallisepticum and M. synoviae - respiratory disease in poultry

### 1.4: RICKETTSIA

- 1.4.1: Rickettsiaceae
  - 1.4.1.1: R. prowazekii - Typhus fever
  - 1.4.1.2: R. typhi - murine typhus in man
  - 1.4.1.3: R. rickettsii - Rocky Mountain Spotted Fever
  - 1.4.1.4: Coxiella Burnetii - Q Fever in cattle, sheep, goats, birds, and man
  - 1.4.1.5: Cowdria ruminatum - Heartwater in cattle
- 1.4.2: Anaplasmataceae
  - 1.4.2.1: A. marginale and A. centrale - Anaplasmosis in cattle
  - 1.4.2.2: A. ovis - Anaplasmosis in sheep
  - 1.4.2.3: Haemobartonella felis - Hemobartonellosis in cats (Feline Infectious Anemia)
  - 1.4.2.4: Haemobartonella canis - Hemobartonellosis in dogs
  - 1.4.2.5: Eperythrozoon - parasites which attack red blood cells in various animals

### 1.5: CHLAMYDIACEAE

- 1.5.1: C. psittaci - Psittacosis - a febrile pulmonary disease in man and birds
  - 1.5.1.1: also causes Sporadic Bovine Encephalomyelitis and polyarthrititis in cattle
  - 1.5.1.2: also causes Epizootic Abortion in cattle and sheep
  - 1.5.1.3: also causes pneumonia in cattle and sheep
  - 1.5.1.4: also causes Feline Pneumonitis in cats
- 1.5.2: C. trachomatis - Venereal disease in man

### 1.6: SPIROCHAETALE

- 1.6.1: Leptospira spp.

- 1.6.1.1: *L. canicola*, *L. grippotyphosa*, *L. hardjo*,  
*L. icterohaemorrhagiae*
- 1.6.1.2: *L. pomona* - all cause disease in various  
species
- 1.6.2: *Treponema* SPP.
- 1.6.2.1: *T. hyodysenteriae* - Swine Dysentery
- 1.6.2.2: *T. pallidum* - Syphilis in man
- 1.6.3: *Borrelia* spp.
- 1.6.3.1: *B. anserina* O Avian borreliosis or  
spirochaetosis in birds

## 1.7: FUNGAL DISEASES

- 1.7.1: *Aspergillus fumigatus* - brooder  
pneumonia in poultry
- 1.7.2: *Blastomyces dermatitidis* - pulmonary  
infection in animals and man
- 1.7.3: *Candida albicans* - Thrush in birds, cats,  
cattle, swine and man
- 1.7.4: *EPIDERMOPHYTON* SPP.
- 1.7.4.1: *E. floccosum* - Athletes foot in man
- 1.7.5: *HISTOPLASMA* SPP.
- 1.7.5.1: *H. capsulatum* - systemic fungal infection  
in many species
- 1.7.6: *MICROSPORUM* SPP.
- 1.7.6.1: *M. canis* - ringworm in dogs, cats, man,  
cattle
- 1.7.6.2: *M. gypseum* - ringworm in dogs, cats,  
horses, man
- 1.7.7: *TRICHOPHYTON* SPP.
- 1.7.7.1: *T. rubrum* - ringworm in dogs, primates,  
and man
- 1.7.7.2: *T. equinum* and *T. quinckeanum* - ringworm  
in horses
- 1.7.8: MYCOTOXICOSES (Moldy feed) caused by numerous  
filamentous fungi
- 1.7.8.1: Aflatoxins, Mycotoxins, *Aspergillus*  
toxins

## 2: PARASITES

### 2.1: PROTOZOA

#### 2.1.1: AMEBA

- 2.1.1.1: *Entamoeba histolytica* - Amebic dysentery  
in dogs, cats, pigs and man

#### 2.1.2: BABESIA SPP.

- 2.1.2.1: *Babesia bigemina* and *B. bovis* are major  
causes babesiosis in cattle  
(babesiosis also known as Texas fever,  
Tick Fever, Prioplasmosis)
- 2.1.2.2: *B. argentina*, *B. Divergens*, and *B. major*  
also cause babesiosis in cattle
- 2.1.2.3: *B. canis* and *B. Gigsoni* - cause  
babesiosis in dogs

- 2.1.2.4: *B. equi* and *B. caballi* cause babesiosis in horses
- 2.1.2.5: *B. motasi* and *B. ovis* - cause babesiosis in horses
- 2.1.2.6: *B. trautmanni* - babesiosis in pigs
- 2.1.2.7: *B. felis* - babesiosis in cats
- 2.1.3: COCCIDIA
- 2.1.3.1: EIMERIA SPP.  
*E. tenella*, *E. necatrix*, *E. brunetti*, *E. acervulina*, *E. maxima* in chickens  
*E. bovis*, *E. zuernii* in cattle
- 2.1.3.2: ISOSPOORA SPP.  
*I. suis* - seine
- 2.1.3.3: SARCOYSTIS SPP.  
*S. tenella* - infects sheep  
*S. blanchardi*, *S. fayeri*, and *S. fusiformis* - infect cattle  
*S. miescheriana* - infects swine
- 2.1.3.4: TOXOPLASMA GONDII  
wide spread distribution, especially in cats, swine, sheep, humans  
causes abortion, birth defects, deafness
- 2.1.3.5: CRYPTOSPORIDIUM SPP.  
cause diarrhea in cattle, swine, sheep, birds, and man  
A component of AIDS complex
- 2.1.4: GIARDIA SPP.
- 2.1.4.1: *G. lamblia* - infects man
- 2.1.4.2: *G. canis* - infects dogs
- 2.1.4.3: *G. cati* - infects cats
- 2.1.4.4: *G. bovis* - infects cattle
- 2.1.5: LEISHMANIA SPP.
- 2.1.5.1: *L. donovani* - visceral leishmania in man, dogs, cats, cattle sheep
- 2.1.5.2: *L. tropica* - cutaneous leishmania in man, dogs, and rodents
- 2.1.5.3: *L. braziliensis* - American leishmaniasis in man, dogs, and cats
- 2.1.6: PLASMODIUM SPP.
- 2.1.6.1: *Plasmodium falciparum* - malaria in man
- 2.1.6.2: *P. malariae*, *P. vivax*, and *P. ovale* - malaria in man
- 2.1.6.3: *P. gallinaceum* - avian malaria
- 2.1.6.4: numerous *Plasmodium* spp. cause malaria in man
- 2.1.7: PNEUMOCYSTOSIS SPP.
- 2.1.7.1: *P. carinii* - cause of pneumonia in man, dogs, horses, swine, goats
- 2.1.7.2: A component of the AIDS complex
- 2.1.8: THEILERIA SPP.
- 2.1.8.1: *T. parva*, *T. annulata*, *T. mutans*, *T. lawrencei* and *T. cervi*

- all cause East Coast Fever in cattle, buffalo and deer
- 2.1.8.2: *T. hirci* and *T. ovis* infect sheep
- 2.1.9: TRITRICHOMONAS SPP.
- 2.1.9.1: *T. vaginalis* - a venereal disease of man
- 2.1.9.2: *T. foetus* - causes trichomoniasis, a genital infection of cattle
- 2.1.9.3: *Trichomonas gallinae* - causes trichomoniasis, a G.I. infection in birds
- 2.1.10: TRYPANOSOMA SPP.
- 2.1.10.1: *T. cruzi* - Chagas disease in man
- 2.1.10.2: *T. congolense* -- Trypanosomiasis in cattle, horses, pigs, dogs
- 2.1.10.3: *T. rhodesiense* and *T. gambiense* - sleeping sickness in man and antelope
- 2.2: HELMINTHS
- 2.2.1: TREMATODES
- 2.2.1.1: FLUKES
  - Fasciola hepatica* - cattle and sheep
  - F. gigantica* - cattle and sheep
  - Fascioloides magna* - cattle, sheep and swine
  - Dicrocoelium dendriticum* - cattle, sheep, horses, swine, man
- 2.2.1.2: SCHISTOSOMIASIS
  - Schistosoma japonicum*, *S. hematobium*, *S. mansoni*, *S. intercalatum* - man
  - S. bovis*, *S. spindale*, *S. mattheei* - cattle, sheep, goat, horse
  - S. nasalis*, *S. indium* - cattle, sheep, goats
- 2.2.1.3: PARAGONIMIASIS (SALMON POISONING)
  - Paragonimus westermani* - man
  - P. kellicotti* - mink, dog, cat, pig
- 2.2.2: CESTODES
- 2.2.2.1: TAPEWORMS
  - Taenia saginata*, and *T. solium* - man (cysticercus)
  - Echinococcus granulosus*, and *E. multilocularis* - man, dog
  - Taenia hydatigena*, *T. ovis* - dog
  - T. pisiformis* - dog and cat
  - Dipylidium caninum* - dog and cat
  - Anoplocephala magna*, *A. perfoliata* - horses
- 2.2.2.2: ECHINOCCUS SPP.
- 2.2.2.3: DIPHYLLOBOTHRIUM SPP.
- 2.2.2.4: SPIROMETRA SPP.
- 2.2.2.5: FASCIOLA SPP.
- 2.2.3: NEMATODES
- 2.2.3.1: FILARIAL PARASITES
  - Dirofilaria immitis* - heartworm in dogs
- 2.2.3.2: HOOKWORMS

- A. duodenale and Necator americanus -  
 hookworm in man  
 A. caninum, A. braziliense - dogs and  
 cats  
 Uncinaria stenocephala - dogs  
 Bunostomum phlebotomum - cattle  
 B. trigonocephalum - sheep and goats  
 Globecephalus urosubulatus - swine  
 2.2.3.3: KIDNEY WORMS  
 Dictyophyma renale - dog  
 2.2.3.4: LUNGWORMS  
 Dictyocaulus viviparus - lungworm in  
 cattle  
 D. filaria - lungworm in sheep, goat,  
 cattle  
 Muellerium capillaris - lungworm in sheep  
 Metastrongylus apri, M. pudendotectus, M.  
 salmi - swine  
 2.2.3.5: NODULAR WORMS  
 Oesophagostomum denatum - swine  
 O. radiatum, and O. columbianum -  
 cattle, sheep, goats  
 2.2.3.6: ONCHOCERIASIS  
 Onchocerca volvulus - blindness in humans  
 2.2.3.7: PINWORMS  
 Enterobius vermicularis - man  
 Oxyuris equi - horses  
 Skrjabinema ovis - sheep and goats  
 2.2.3.8: ROUNDWORMS  
 Ascaris lumbricoides - roundworms in man,  
 swine  
 Toxocara canis - dogs  
 Toxocara cati - cats  
 Parascaris equorum - horse  
 Ascaridia galli - chickens  
 2.2.3.9: SPIROCERCAS  
 Spriocerca lupi - dogs  
 2.2.3.10: STOMACH WORMS  
 Habronema, H. majus, H. megastoma -  
 horses  
 2.2.3.11: STRONGYLES  
 Strongylus vulgaris, S. equinus, S.  
 edentatus - horses  
 2.2.3.12: STRONGYLOIDS  
 Strongyloides westeri - horses  
 S. stercoralis - man  
 S. ransomi - swine  
 S. canis - dogs  
 S. tumefaciens - cats  
 2.2.3.13: TRICHINA  
 Trichinella spiralis - trichinella in  
 swine and man  
 2.2.3.14: TRICHOSTRONGYLES

- Ostertagia ostertagi - cattle
- Haemonchus placei - cattle
- Trichostrongylus axei - cattle
- Cooperia punctata - cattle
- Haemonchus contortus, Cuperia curticei - sheep
- Ostertagia circumcincta - sheep
- Trichostrongylus colubriformis - equine, swine, cattle, sheep
- Nematodirus filicollis - cattle and sheep
- Hyostrogylus rubidus - swine
- 2.2.3.15: WHIPWORMS
  - Trichuris ovis - cattle, sheep, goats
  - Trichuris suis - swine
  - T. trichiura - man
  - T. vulpis - dogs
- 2.3: ARTHROPODS
- 2.3.1: ACARIASIS
- 2.3.1.1: Demodex folliculorum - mange in dogs, cats, cattle, swine, sheep, man
- 2.3.1.2: Demodex phylloides - mange in swine
- 2.3.1.3: Dermacentor andersoni - wood tick
- 2.3.1.4: Dermanyssus gallinae - red mite in poultry
- 2.3.1.5: Ixodes holocyclus - Australian tick
- 2.3.1.6: Notoedres cati - cat mange
- 2.3.1.7: Otobius megnini - spinose ear tick
- 2.3.1.8: Ostodectes cynotis - ear mite in dog, cat
- 2.3.1.9: Psoroptes communis - scab in cattle, sheep, horses
- 2.3.1.10: Sarcoptes scabiei, S. canis - mange in dogs
- 2.3.2: DIPTERA
- 2.3.2.1: BOTFLIES
  - Gasterophilus intestinalis - equine botfly
  - Gasterophilus hemorrhoidalis - equine nose botfly
  - Gasterophilus nasalis - equine chinfly
  - Gasterophilus pecorum - European botfly
  - Gasterophilus inermis - botfly
  - Oestrus ovis - sheep botfly
- 2.3.2.2: FLEAS
  - Otenocephalides canis - dog flea
  - Ctenocephalides felis - cat flea
- 2.3.2.3: FLIES
  - Chrysops spp. - deer flies
  - Fannia spp. - little house flies
  - Haematobia irritans - horn flies
  - Haematobia irritans exigua - buffalo fly (similar to horn fly)
  - Hermetia illucens - black soldier fly
  - Hybomitra spp. common fly

- Hydrotaea irritans - head flies  
 Ophyra spp. - dump flies  
 Melophagus ovinus - sheep ked  
 Musca autumnalis - face flies  
 Musca domestica - house fly  
 Muscina spp. - false stable flies  
 Simulium spp. - black flies (no-see-ums)  
 Stomoxys calcitrans - stable flies  
 Tabanus spp. - horse flies
- 2.3.2.4: GRUBS  
 Hypoderma lineatum, H. bovis - Heel fly, cattle grub  
 Calitroga americana - screw-worm fly  
 Dermatobia hominis - cutaneous myiasis in man, cattle sheep, dogs, cats  
 Cochliomyia hominivorax - blow fly
- 2.3.2.5: LICE  
 Damalinia bovis - cattle biting louse  
 Anoplura spp. - cattle louse  
 Haematopinus eurysternus - shortnosed cattle louse  
 Linognathus vituli - longnosed cattle louse  
 Solenoptes capillatus - little blue cattle louse  
 Haematopinus suis - swine lice  
 Haematopinus asini - horse sucking louse  
 Trichodectes canis - dog louse  
 Felicola subrostrata - cat louse
- 2.3.2.6: MOSQUITOES  
 Aedes spp.  
 Anopheles spp.  
 Culex spp.  
 Culiseta spp.  
 Psorophora spp.

<u>Disease</u>	<u>Pathogen(s)</u>
Malaria	Plasmodium falciparum P. vivax P. malariae P. ovale P. berghei etc.
Chagas' Disease	Trypanosoma cruzi
African Trypanosomiasis	Trypanosoma gambiense T. rhodesiense T. brucei etc.
Leishmaniasis	Leishmania donovani



	<i>L. infantum</i> <i>L. tropica</i> <i>L. mexicana</i> <i>L. braziliensis</i> <i>L. chagasi</i> etc.
Leprosy	<i>Mycobacterium leprae</i>
Tuberculosis	<i>Mycobacterium tuberculosis</i>
Filariasis	<i>Brugia malayi</i> <i>B. timori</i> <i>Onchocerca volvulus</i> <i>Wuchereria bancrofti</i>
Schistosomiasis	<i>Schistosoma mansoni</i> <i>S. japonicum</i>
Leptospirosis	<i>Leptospira interrogans</i> <i>L. icterohaemorrhagiae</i> <i>L. hebdomadis</i> <i>L. pomona</i> etc.
Plague	<i>Yersinia pestis</i>
Typhoid Fever	<i>Salmonella typhi</i>
Cholera	<i>Vibrio cholerae</i>
Diphtheria	<i>Corynebacterium diphtheriae</i>
Lyme Disease	<i>Borrelia burgdorferi</i>
Pneumonia/bronchitis	<i>Streptococcus pneumoniae</i> <i>Mycoplasma pneumoniae</i> <i>Branhamella catarrhalis</i> <i>Bordetella bronchiseptica</i> <i>Haemophilus influenza</i>
Urethritis	<i>Mycoplasma hominis</i> <i>Ureaplasma urealyticum</i>
Giardia	<i>Giardia lamblia</i>
Amoebic dysentery	<i>Entamoeba histolytica</i>
Syphilis	<i>Treponema pallidum</i>
Chlamydia	<i>Chlamydia trachomatis</i>
Candidiasis	<i>Candida albicans</i>

	<i>C. glabrata</i>
Gonorrhea	<i>Neisseria gonorrhoeae</i>
Toxoplasmosis	<i>Toxoplasma gondii</i>
Tetanus	<i>Clostridium tetani</i>
Caries	<i>Streptococcus mutans</i>
Whooping cough	<i>Bordetella pertussis</i>
Q fever endocarditis	<i>Coxiella burnetti</i>
Anthrax	<i>Bacillus anthracis</i>
Brucellosis	<i>Brucella abortus</i>

Numerous modifications and variations of the present invention are possible in light of the above teachings; therefore, within the scope of the appended claims the invention may be practiced otherwise than as particularly described.

## WHAT IS CLAIMED IS:

1. A vaccine for protecting against an organism, comprising:
  - (a) a protein which is capable of eliciting an antibody which recognizes at least one epitope of a native protein present in the organism, said native protein having at least 50% homology with a heat shock protein of *T. cruzi*; and
  - (b) a physiologically acceptable carrier.
2. A vaccine of claim 1 wherein the native protein is derived from a species of *Mycoplasma*, *Mycobacteria*, or *Trypanosoma*, provided that the native protein is not derived from *Trypanosoma cruzi*.
3. A vaccine of claim 2 wherein the native protein is derived from a species of *Mycoplasma* selected from the group consisting of *M. mycoides*, *M. bovis*, *M. bovis genitalium*, *M. bovoculi*, *M. bovirhinis*, *M. dispar*, *M. hyorhinis*, *M. hyosynoviae*, *M. hyopneumoniae*, *M. gallisepticum*, *M. pneumoniae* and *M. synoviae*.
4. A vaccine of claim 2 wherein the native protein is derived from a species of *Mycobacteria* selected from the group consisting of *M. bovis*, *M. leprae* and *M. tuberculosis*.
5. A vaccine of claim 3 wherein the native protein is derived from a species of *Mycoplasma* selected from the group consisting of *M. hyopneumoniae* and *M. gallisepticum*.

6. A process for protecting a host against an organism comprising:

administering an effective amount of a protein capable of eliciting an antibody which recognizes at least one epitope of a native protein present in the organism said native protein having at least 50% homology with a *T. cruzi* heat shock protein.

7. A process of claim 6 wherein the native protein is derived from a species of *Mycoplasma*, *Mycobacteria*, or *Trypanosoma*, provided that the native protein is not derived from *Trypanosoma cruzi*.

8. A process of claim 7 wherein the native protein is derived from a species of *Mycoplasma* selected from the group consisting of *M. mycoides*, *M. bovis*, *M. bovis genitalium*, *M. bovis oculi*, *M. bovis rhinis*, *M. dispar*, *M. hyorhinis*, *M. hyosynoviae*, *M. hyopneumoniae*, *M. gallisepticum*, *M. pneumoniae*, and *M. synoviae*.

9. A process of claim 7 wherein the native protein is derived from a species of *Mycobacteria* selected from the group consisting of *M. bovis*, *M. leprae*, and *M. tuberculosis*.

10. A process of claim 8 wherein the native protein is derived from a species of *Mycoplasma* selected from the group consisting of *M. hyopneumoniae* and *M. gallisepticum*.

11. A process for determining an organism in a host comprising:

contacting a sample derived from a host containing or suspected of containing an organism with an antigen which is recognized by an antibody elicited in response to a protein present in the organism, said protein

having at least 50% homology with a heat shock protein of *T. cruzi*; and determining antibody in said sample bound by said antigen.

12. A process for determining an organism in  
5 a host, comprising:

contacting a sample derived from a host containing  
an organism or suspected of containing an organism with  
an antibody or fragment of said antibody, said antibody  
recognizing at least one epitope of a native protein  
10 present in the host, said native protein having at  
least 50% homology with a heat shock protein of *T. cruzi*; and

determining protein present in said organism bound  
to said antibody.

13. A process of claim 12 wherein the native  
15 protein is derived from a species of *Mycoplasma*,  
*Mycobacteria*, or *Trypanosoma*, provided that the native  
protein is not derived from *Trypanosoma cruzi*.

14. A process of claim 13 wherein the native  
20 protein is derived from a species of *Mycoplasma*  
selected from the group consisting of *M. mycoides*, *M.*  
*bovis*, *M. bovigenitalium*, *M. bovoculi*, *M. bovirhinis*,  
*M. dispar*, *M. hyorhinis*, *M. hyosynoviae*, *M.*  
*hyopneumoniae*, *M. gallisepticum*, *M. pneumoniae* and *M.*  
25 *synoviae*.

15. A process of claim 13 wherein the native  
protein is derived from a species of *Mycobacteria*  
selected from the group consisting of *M. bovis*, *M.*  
*leprae*, and *M. tuberculosis*.

16. A process of claim 14 wherein the native protein is derived from a species of *Mycoplasma* selected from the group consisting of *M. hyopneumoniae* and *M. gallisepticum*.

5           17. A recombinant sequence of nucleic acid encoding the heat shock proteins of *M. hyopneumoniae* and *M. gallisepticum* as depicted in figures 5 and 11, respectively.

FIGURE 1-1

Translation of clone pFP70-47

```

-----aal-----pUC9 sequence-----
                met thr met ile thr pro ser leu
      TTCACACAGGAAACAGCT ATG ACC ATG ATT ACG CCA AGC TTG
9-----| |T.cruzi sequence----->
ala ala gly arg arg ile pro lys lys thr ala gly lys lys
GCT GCA GGT CGA CGG ATC CCC AAA AAA ACA GCA GGA AAG AAG
23
lys lys met thr tyr glu gly ala ile gly ile asp leu gly
AAG AAA ATG ACG TAC GAG GGA GCC ATC GGC ATC GAT CTC GGC
37
thr thr tyr ser cys val gly val trp gln asn glu arg val
ACA ACT TAC TCG TGC GTT GGT GTT TGG CAG AAC GAG CGC GTG
51
glu ile ile ala asn asp gln gly asn arg thr thr pro ser
GAG ATC ATT GCG AAC GAC CAG GGC AAC CGC ACA ACG CCG TCG
65
tyr val ala phe thr asp thr glu arg leu ile gly asp ala
TAC GTG GCG TTC ACC GAC ACG GAG CGT CTG ATC GGT GAT GCC
79
ala lys asn gln val ala met asn pro thr asn thr val phe
GCG AAG AAC CAG GTT GCG ATG AAC CCG ACG AAC ACC GTC TTC
93
asp ala lys arg leu ile gly arg lys phe ser asp pro val
GAC GCG AAG CGC CTC ATT GGG CGG AAG TTC AGC GAC CCC GTT
107
val gln ser asp met lys his trp pro phe lys val ile thr
GTG CAG TCG GAC ATG AAG CAC TGG CCC TTC AAG GTC ATC ACG
121
lys gly asp asp lys pro val ile gln val gln phe arg gly
AAG GGC GAC GAC AAG CCG GTG ATC CAG GTG CAG TTC CGC GGC
135
glu thr lys thr phe asn pro glu glu val ser ser met val
GAG ACA AAG ACG TTC AAC CCG GAG GAG GTG AGC TCG ATG GTG
149
leu ser lys met lys glu ile ala glu ser tyr leu gly lys
CTG TCA AAG ATG AAG GAG ATT GCG GAG TCG TAC CTG GGC AAG

```

FIGURE 1-2

163

gln val lys lys ala val val thr val pro ala tyr phe asn  
CAG GTG AAG AAG GCC GTG GTG ACT GTG CCC GCG TAC TTC AAC

187

asp ser gln arg gln ala thr lys asp ala gly thr ile ala  
GAC TCC CAG CGG CAG GCG ACG AAG GAT GCC GGC ACG ATC GCG

201

gly met glu val leu arg ile ile asn glu pro thr ala ala  
GGG ATG GAG GTG CTG CGC ATC ATC AAT GAG CCG ACA GCT GCC

215

ala ile ala tyr gly leu asp lys val glu asp gly lys glu  
GCC ATT GCG TAC GGC CTG GAC AAA GTG GAG GAC GGC AAG GAG

239

arg asn val leu ile phe asp leu gly gly gly thr phe asp  
CGC AAT GTG CTC ATC TTT GAC CTT GGC GGC GGC ACG TTT GAT

253

val thr leu leu thr ile asp gly gly ile phe glu val lys  
GTC ACG CTG CTG ACG ATC GAC GGT GGC ATC TTT GAG GTG AAG

267

ala thr asn gly asp thr his leu gly gly glu asp phe asp  
GCG ACG AAC GGC GAC ACG CAC CTG GGC GGC GAG GAC TTT GAC

281

asn arg leu val ser his phe thr asp glu phe lys arg lys  
AAC CGC CTC GTG TCG CAC TTC ACG GAC GAG TTC AAG CGC AAG

295

asn lys gly lys asp leu thr thr ser gln arg ala leu arg  
AAC AAG GGC AAG GAC CTG ACG ACA AGC CAG CGC GCC CTC CGC

309

arg leu arg thr ala cys glu arg ala lys arg thr leu ser  
CGC CTC CGC ACC GCC TGC GAG CGC GCC AAG CGC ACG CTG TCG

323

ser ala ala gln ala thr ile glu ile asp ala leu phe asp  
TCC GCG GCA CAG GCG ACG ATT GAG ATC GAC GCG CTG TTT GAC

337

asn val asp phe gln ala thr ile thr arg ala arg phe glu  
AAC GTG GAC TTC CAG GCA ACC ATC ACT CGC GCC CGC TTC GAG



3/32

FIGURE 1-3

351

glu leu cys gly asp leu phe arg gly thr leu gln pro val  
GAG CTC TGC GGC GAC CTC TTC CGA GGG ACG CTG CAG CCG GTG

365

glu arg val leu gln asp ala lys met asp lys arg ala val  
GAG CGT GTG CTC CAG GAC GCC AAG ATG GAC AAG CGT GCC GTG

379

his asp val val leu val gly gly ser thr arg ile pro lys  
CAC GAC GTG GTG CTC GTC GGC GGC TCC ACC CGC ATT CCA AAG

393

val met gln leu val ser asp phe phe gly gly lys glu leu  
GTG ATG CAG CTG GTG TCT GAC TTT TTC GGT GGC AAG GAA CTG

407

asn lys ser ile asn pro asp glu ala val ala tyr gly ala  
AAC AAG AGC ATC AAC CCT GAT GAG GCT GTG GCG TAC GGT GCC

421

ala val gln ala phe ile leu thr gly gly lys ser lys gln  
GCC GTG CAG GCC TTC ATC CTG ACG GGC GGC AAG AGC AAG CAG

435

thr glu gly leu val leu leu asp val thr pro leu thr leu  
ACG GAG GGC CTC GTG CTG CTC GAC GTG ACC CCG CTG ACG CTT

449

gly ile glu thr ala gly gly val met thr ser leu ile lys  
GGC ATC GAG ACG GCG GGT GGC GTC ATG ACG TCG CTG ATC AAG

463

arg asn thr thr ile pro thr lys lys ser gln ile phe ser  
CGC AAC ACG ACG ATT CCG ACC AAG AAA AGC CAG ATC TTC TCG

477

thr tyr ala asp asn gln pro gly val his ile gln val phe  
ACG TAC GCG GAC AAC CAG CCG GGC GTG CAC ATC CAG GTC TTT

491

glu gly glu arg ala met thr lys asp cys his leu leu gly  
GAG GGG GAG CGT GCG ATG ACG AAG GAC TGC CAC CTG CTC GGC

515

thr phe asp leu ser gly ile pro pro ala pro arg gly va  
ACA TTC GAC CTG TCC GGC ATC CCG CCG GCG CCG CGC GGT GTG

SUBSTITUTE SHEET

## FIGURE 1-4

529

pro gln ile glu val thr phe asp leu asp ala asn gly ile  
CCC CAG ATT GAG GTT ACC TTT GAC CTC GAC GCC AAC GGC ATC

543

leu asn val ser ala glu glu lys gly thr gly lys arg asn  
CTG AAC GTG TCC GCG GAG GAG AAG GGC ACC GGC AAG CGC AAC

557

gln ile val ile thr asn asp lys gly arg leu ser lys ala  
CAG ATT GTC ATC ACG AAC GAC AAG GGC CGC CTG AGC AAG GCG

571

asp ile glu arg met val ser glu ala ala lys tyr glu ser  
GAC ATT GAG CGC ATG GTG TCC GAG GCT GCC AAG TAC GAG TCG

585

gln asp lys glu gln arg glu arg ile asp ala lys asn gly  
CAG GAC AAG GAA CAG CGC GAG CGC ATT GAC GCA AAG AAC GGT

599

leu glu asn tyr ala phe ser val lys asn thr val asn glu  
CTT GAG AAC TAC GCA TTT TCG GTG AAG AAC ACC GTA AAC GAG

613

pro asn val ala gly lys ile glu glu ala asp lys asn thr  
CCG AAC GTC GCT GGC AAG ATT GAG GAG GCC GAC AAG AAC ACG

627

ile thr ser ala val glu glu ala leu gln trp leu asn asn  
ATT ACG AGT GCC GTG GAG GAG GCG CTG CAA TGG CTG AAC AAC

5/32

FIGURE 1-5

641

asn gln glu ala ser lys glu glu tyr glu his arg gln lys  
AAC CAG GAG GCC AGC AAG GAG GAG TAC GAG CAC CGC CAG AAG

655

glu leu glu asn leu cys thr pro ile met thr lys met tyr  
GAG CTG GAG AAC CTG TGC ACG CCC ATC ATG ACG AAG ATG TAC

669

gln gly met gly ala gly gly gly met pro gly gly met pro  
CAG GGC ATG GGC GCG GGC GGC GGT ATG CCC GGA GGT ATG CCT

683

gly gly met pro gly gly met pro gly gly ala asn pro ser  
GGT GGA ATG CCC GGG GGC ATG CCT GGT GGC GCG AAC CCG TCG

697

ser ser ser gly pro lys val glu glu val asp OP  
TCT TCG TCA GGA CCG AAG GTG GAG GAA GTG GAC TGA GAGCGCATCC

CTGAAGATGTTCCCATGGCGGCGTCTGCTCGCGAACGAATAACCCGTTGGTTTTCTCC

CTTG TAGAGCGTAGAGGTCTGCGACAAACCCAGCCGCCATCACTATTTTATTATTGG

TTTTTTTCCCTCTCCATTATTATTATTATTATTATTATTATTATTACGGTTGTTATTT

GTATTGTCATTGCGATGGCACTTGCTGCTGTTGAGGGCACCACGGTTGCCTCTGCCATT

TTTGTTGCTGACTGACGCCTGTGTGCGTCTCCTTGTTACCGCCGGCTTCCTTTCTCCT

TTCTCCCCCGCTCCTTCGCCCTGT

FIGURE 2-1

CLUSTERED PAIR-WISE ALIGNMENT listed in clustered order,  
in 'identity (no translation)' alphabet of:

- |                     |                      |
|---------------------|----------------------|
| 1. Mhyhsp70 (1-600) | 7. x170 (1-647)      |
| 2. Bmehsp70 (1-605) | 8. humhsp70 (1-641)  |
| 3. dnaK (1-638)     | 9. chkhsp70 (1-635)  |
| 4. tc70kd (1-669)   | 10. mzehsp70 (1-646) |
| 6. rathsp70 (1-646) | 11. smahsp70 (1-620) |

```

1  makeIIlGIDLGTINSvVAiiEnqkPvV  leNPnGkRTTPS VVAFKNnEeiV
    || ||||| || | | | | | | | | | |
1  MsKII GIDLGTINSCVAvlEGgePkV  ipNPEGnRTTPS VVAFKNGErqV
    | ||| ||||| || | | | | | | | |
1  MgKII GIDLGTINSCVAimdGttPRV  leNaEGdRTTPSiiaytqDGEtLV
    | ||||| || | | | | | | | |
1  MTYEGAI GIDLGTTYSCV  GVWQNERVEIIANDQGNRTTPS YVAFtDtERLI
    ||||| ||||| ||||| ||||| ||||| |||||
1  MTYEGAI GIDLGTTYSCV  GVWQNERVEIIANDQGNRTTPS YVAFtDsERLI
    | | ||||| ||||| ||||| ||||| |||||
1  MsKGpA VGIDLGTTYSCV  GVFQHGKVEIIANDQGNRTTPS YVAFtDTERLI
    || | ||||| ||||| ||||| ||||| |||||
1  MatKGvA VGIDLGTTYSCV  GVFQHGKVEIIANDQGNRTTPS YVAFtDTERLI
    || | ||||| ||||| ||||| ||||| |||||
1  MA KaaA VGIDLGTTYSCV  GVFQHGKVEIIANDQGNRTTPS YVAFtDTERLI
    ||||| ||||| ||||| ||||| ||||| |||||
1  msgkGPAIGIDLGTTYSCV  GVFQHGKVEIIANDQGNRTTPS YVAFtDTERLI
    ||||| ||||| ||||| ||||| ||||| |||||
1  makseGPAIGIDLGTTYSCV  GLwQHDrVEIIANDQGNRTTPS YVgFtDTERLI
    || ||||| ||||| ||||| ||||| |||||
1  fQHgkVEIIANDQGNRTTPS YVaFtDsERLI

```

FIGURE 2-2

```

52 GdaAKRQleTNP eaIaSiKR                               lMG
   |  ||||  |||  |  |||                               |||
50 GevAKRQaiTNP NTIiSvKR                               hMG
   |  |||||  |||  ||  ||
51 GqpAKRQAvTNPqNTlFaiKRLIGRrFqDeeVQrDvsimPFKiiadnGD
   |  ||  |  ||  ||  ||  ||  ||  ||  ||  ||  ||  ||  ||  ||
52 GDAAKNQVAMNPTNTIVFDAKRLIGRKFSDpVVQSDMKHWPFK  ViTKGDDKP
   |||||||||||||||||||||||||||||||||||||||  |||||||
52 GDAAKNQVAMVPTNTIVFDAKRLIGRKFSDsVVQSDMKHWPFK  VVTKGDDKP
   |||||||||||||||||||||||||||  ||  |||||||
52 GDAAKNQVAMNPTNTIVFDAKRLIGRrFdDaVVQSDMKHWPF  mVV nDaGrP
   |||||||||||  |||||||||||  ||  |||  ||  |||  ||  ||
53 GDAAKNQVAMNPQNTIVFDAKRLIGRKFhDPVVQcDlKHWPF  QVV sDeGKP
   |||||||||  |||||||||||  |||||  |||||||  ||  ||  ||
52 GDAAKNQVALNPQNTIVFDAKRLIGRKFgDPVVQcDlKHWPF  QV iNDGdKP
   |||||||||  ||  ||  |||||||||  ||  ||  ||  |||  ||
53 GDAAKNQVAMNPTNTiFDAKRLIGRKYdDPtVQSDMKHWPF  RV vNeGgKP
   |||||||||||  ||||  |||||||  ||  |||  ||  ||  ||
54 GDAAKNQVAMNPTNTIVFDAKRLIGRREssPaVQSSMKlWP  sR hlgIGdKP
   ||  |||||||||||  |||||||  ||  |||  ||  ||  ||
32 GDgAKNQVAMNPTNTIVFDAtRLIGRREfpdPsVQScMKhWP  fevtqvGgKl

```

# SUBSTITUTE SHEET

9/32

FIGURE 2-4

```

128 eATKnAGKIAGLqVERIINEPTAAALafGL dK TekemkVLVYDLGGGTFD
    ||| ||||| ||||| ||||| ||| | | ||||| |||||
126 QATKDAGKIAGLEVERIINEPTAAALAYGL eK TdedqTVLVYDLGGGTFD
    ||||| ||||| ||||| ||||| ||| | | ||||| |||||
152 QATKDAGrIAGLEVkRIINEPTAAALAYGLD K gtgnRTiaVYDLGGGTFD
    ||||| || || || ||||| ||||| ||||| | |||||
157 QATKDAGTIAGmEVLRIINEPTAAAIAYGLD KvedGKERNVLIFDLGGGTFD
    ||||| ||||| ||||| ||||| ||||| | ||||| |||||
157 QATKDAGTIAGLEVLRIINEPTAAAIAYGLD KadeGKERNVLIFDLGGGTFD
    ||||| ||||| ||||| ||||| ||||| | ||||| |||||
156 QATKDAGTIAGLNVLRIINEPTAAAIAYGLD K kvGaERNVLIFDLGGGTFD
    ||||| ||||| ||||| ||||| ||||| | ||||| |||||
157 QATKDAGVLAGLNiLRIINEPTAAAIAYGLD K garGEqNVLIFDLGGGTFD
    ||||| ||||| ||||| ||||| ||||| || ||||| |||||
156 QATKDAGVIAGLNVLRIINEPTAAAIAYGLD rTgkGErNVLIFDLGGGTFD
    ||||| ||||| ||||| ||||| ||||| || ||||| |||||
157 QATKDAGtItGLNVMRIINEPTAAAIAYGLDKKgTraGEKNVLIFDLGGGTFD
    ||||| | ||||| ||||| ||||| ||||| | ||||| |||||
158 QATKDAGvIAGLNVMRIINEPTAAAIAYGLDKKaTssGEKNVLIFDLGGGTFD
    ||||| ||||| ||||| ||||| ||||| || ||||| |||||
135 QATKDAGaIAGLNVLRIINEPTAAAIAYGLDKK vgGErNVLIFDLGGGTFD

```

10/32

FIGURE 2-5

```

178 VS  vLELsgG  tFEVlsTsGDNhLGGDDwDneIvnoLV  kkiKEvydfDpks
      ||  |||  |  |||  |  |||  |||||  |  |  ||  ||  |
176 VSI  LELgDG  vFEVrATaGDNrLGGDDFDqvIIIdYLVaEFKKE  nGvDLsk
      ||  ||  |||  ||  ||  |||  |||  |  |||  ||||  |  ||
202 iSIieidevDGektFEVLATNGDTHLGGEDFDsRLInYLVaEFKK  dqGiDLrn
      ||  |||  |||||  |||||  ||  ||  |||  ||  ||
209  VTLLTIDG  GIFEVKATNGDTHLGGEDFDNRLVsHF'TdEEFKRKNGKGDltt
      |||||  |||||  |||||  |||||  |||||  |||||  |||||  |||||
209  VTLLTIDG  GIFEVKATNGDTHLGGEDFDNRLVaHF'TeEFK RKNGKGDLSs
      |  |||  |||||  |  |||||  |||||  |  |  |||||  ||  |
207  VSILTI  eDGIFEVKsTAGDTHLGGEDFDNRMVNHGlaEEFKRK  HKKDISe
      |||||  |||||  |||||  |||||  |||||  |||||  |||||  |||||
208  VSILTI  DDGIFEVKATAGDTHLGGEDFDNRLVNHFVEEFK RK HKKDIgQ
      |||||  |||||  |||||  |||||  |||||  |||||  |||||  |||||
207  VSILTI  DDGIFEVKATAGDTHLGGEDFDNRLVNHFVEEFK RK HKKDI sQ
      |||||  |||||  |||||  |||||  |||||  |||||  |||||  |||||
210  VSILTI  EDGIFEVKsTAGDTHLGGEDFDNRMVNrFVEEFK gK HKrDnaG
      |||||  |  |||||  |||||  |||||  |||||  ||  |||  |  |  |
211  VSILTI  EeGIFEVKaTAGDTHLGGEDFDNRMVNHFVqEFK rK nKKDI sG
      |||||  |  |||||  |||||  |||||  |||||  ||||  ||  |  ||  |
187  VSILTI  EdGIFEVKsTAGDTHLGGEDFDNRMVdHFVKEFq kK ynKDI rG

```



FIGURE 2-6

```

227 DKMALtRLKeeAEKtKinLSnqsvST vSLPFlgmGknGPinveLE LkRseF
    ||||| ||| ||| | || || ||||| | || || ||| |
225 DKMALQRLKdAAEKAKkdLS gvtSTqiSLPFITAGeaGP lHLEvsLsRAKF
    | |||| ||||| || | || ||||| || | |||
255 DplAmQRLKeAAEKAKieLS SAqqTdvnLPyITADAtGP kHmnikvTRAKl
    | || | | | || || || || |||||
259 sqRALRRLRTACERAKRTLS SA AQATIEIDA LFdNvDFQATITRARF
    ||||| ||||| || || ||||| |||||
259 NlRALRRLRTACERAKRTLS SA AQATIEIDA LFENIDFQATITRARF
    | || ||||| ||||| | || ||||| |||||
256 NKRAVRRLRTACERAKRTLS S StQASIEID SLyEGIDFYTsITRARF
    |||| ||||| ||||| | | ||||| |||||
257 NKRALRRLRTACdRAKRTLS S SsQASIEID SLFEGIDFYTaITRARF
    |||| ||||| ||||| | ||||| |||||
256 NKRAVRRLRTACERAKRTLS S STgASIEID SLFEGIDFYTSITRARF
    ||||| ||||| ||||| ||||| |||||
257 NKRAVRRLRTACERArRTLS S STQASIEID SLFEGIDFYTSITRARF
    | || ||||| ||||| | ||||| |||||
258 NpRALRRLRTACERAKRTLS S tAQItIEID SLFEGIDFtprssRARF
    | ||||| ||||| | ||||| |||||
236 NkRALRRLRTACERAKRTLS S sAQInIEID SLcdGIDFytvitRARF

```

.12/32

FIGURE 2-7

```

278 ekmtAhLidRTrkPivdALKqAkiesASdLDevLLVGGSTRmP AVQsmI
      | | | | | | | | | | | | | | | | | | | | |
275 deLsAgLVerTmaPvrqALKDAGLSASeLDkVILVGGSTRiP AVQdaIKK
      | | | | | | | | | | | | | | | | | | | | |
305 EsLowDLVnRsiePlkvALQDAGLSvSdiDDVILVGGqTRmP mVQ KKV
      | | | | | | | | | | | | | | | | | | | | |
305 EELCGDLFRGTLQPVERVLQDAKMDKRAVHDVVLVGGSTRIPK V MQLV
      | | | | | | | | | | | | | | | | | | | | |
305 EELCGDLFRGTLQPVERVLQDAKMDKRAVHDVVLVGGSTRIPK V MQLV
      | | | | | | | | | | | | | | | | | | | | |
302 EELnaDLFRGTLdPVEKALRDAKLDKSQIHdIVLVGGSTRIPK iQKLL
      | | | | | | | | | | | | | | | | | | | | |
303 EELCSDLFRGTLEPVEKALRDAKLDKSQIHdIVLVGGSTRIPK VQKLL
      | | | | | | | | | | | | | | | | | | | | |
302 EELCSDLFRsTLEPVEKALRDAKLDKaQIHdIVLVGGSTRIPK VQKLL
      | | | | | | | | | | | | | | | | | | | | |
305 EELNaDLFRgTLEPVEKALRDAKLDKgQIgeiVLVGGSTRIPK iQKLL
      | | | | | | | | | | | | | | | | | | | | |
306 EELNmDLFRkcmEPVEKcLRDAKMDKSsvHDvVLVGGSTRIPK VQ qL
      | | | | | | | | | | | | | | | | | | | | |
283 EELNaDLFRgtldPVEKaLRDAKMDKSqiHDiVLVGGSTRIPK VQkLL

```

FIGURE 2-8

```

326 ehtlnkkPnrsiNPDEVVAiGAAIQGGVLaG      eisDVllLLDVTPLtLGIE
      |      ||||| ||||| |||||
326  etggdPhKgVNPDEVVAiGAAIQGGVLTG      DVLDVvLLDVTPLSLGIE
      | | ||||| ||||| |||||
353 aeFFG KEPrKdVNPDEAVAiGAAVQGGVLTG      DVKDVllLLDVTPLSLGIE
      ||| | | ||||| ||||| |||||
354 SDFFGGKElNKsINPDEAVAYGAAVQAFILTGGKSKQTEG LLLDVTPLtLGIE
      ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| |||||
353 SDFFGGKElNKsINPDEA YGAAVQAFILTGGKSKQTEGLllLLDVaPLtLGIE
      ||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| |||||
351 QDFFNGKElNKsINPDEAVAYGAAVQAAILsGDKSENVQDlLLLDVtPLSLGIE
      ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| |||||
352 QDFFNGRElNKsINPDEAVAYGAAVQAAIlMGDKSENVQDlLLLDVAPLSLGLE
      ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| |||||
350 QDFFNGRdLNKsINPDEAVgYGAAVQAAIlMGDKSENVQDlLLLDVAPLSLGLE
      ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| |||||
354 QDFFNGKElNKsINPDEAVAYGAAVQAAIlMGDKSENVQDlLLLDVTPLSLGiE
      ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| |||||
354 QDFFNGKELcKSINPDEAVAYGAAVQAAILSG egnersDlLLLDVTPLSLGLE
      ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| |||||
331 QDFFNGKELnKSINPDEAVAYGAAVQAAILSGdkceavqDlLLLDVaPLSLGLE

```

```

375 TLGGIaTpLIpRNtTIPvtKSQiFSTAeDnQTevTIsVvQGERqlaADNKmL
   | | | | | | | | | | | | | | | | | | | | | | | | | | | |
392 TMGGVfTkLIeRNtTIPTsKSQVFSTAaDsQTAVdIHVLQGERpmsADNKtL
   | | | | | | | | | | | | | | | | | | | | | | | | | | | |
422 TMGGVMtLIakNTTIPTKhSQVFSTAeDNQsAVtIHVLQGERkraADNKsL
   | | | | | | | | | | | | | | | | | | | | | | | | | | | |
426 TAGGVMtSLIKRNtTIPTKKSQIFSTYadNQPGVHIQVFEGERaMTKDCHLL
   | | | | | | | | | | | | | | | | | | | | | | | | | | | |
425 TAGGVMtALIKRNtTIPTKKSQIFSTYSDNQPGVHIQVFEGERtMTKDCHLL
   | | | | | | | | | | | | | | | | | | | | | | | | | | | |
424 TAGGVMtVLIKRNtTIPTKQIQtFtTYSDNQPGVLIQVYEGERAMTKDNNLL
   | | | | | | | | | | | | | | | | | | | | | | | | | | | |
425 TAGGVMtVLIKRNtTIPTKQIQsFtTYSDNQPGVLIQVfEGERAMTKDNNLL
   | | | | | | | | | | | | | | | | | | | | | | | | | | | |
424 TAGGVMtALIKRNsTIPTKQIQiFtTYSDNQPGVLIQVYEGERAMTKDNNLL
   | | | | | | | | | | | | | | | | | | | | | | | | | | | |
427 TAGGVMtALIKRNtTIPTKQIQtFtTYSDNQssVLvQVYEGERAMTKDNNLL
   | | | | | | | | | | | | | | | | | | | | | | | | | | | |
426 TAGGVMtVLIpRNtTIPTKkeQvFsTYSDNQPGVLIQVYEGERArTKDNNLL
   | | | | | | | | | | | | | | | | | | | | | | | | | | | |
404 TAGGVMtALikRNtTIPTKqtQtFtTYSDNQPGVLIQVfEGERALTKDNNLL

```

FIGURE 2-10

427 GRFnLsgIeaAPRGIPQIEVSFsIDvNGittVsAKDkkTgK EQtIT  
| | | | | | | | | | | | | | | | | | | | | |  
425 GRFqLtdIpPAPRGvPQIEVSFDIDkNGIvnVrAKDlgtThK EQaIT  
| | | | | | | | | | | | | | | | | | | | | |  
455 GqFnLdGInPAPRGmPQIEVTFDIDAdGILhVSAKDKnsGK EQkIT  
| | | | | | | | | | | | | | | | | | | | | |  
459 GTFDLSGIPPAPRGVPQIEVTFDILDANGILnVSAEEKGTGKRNQIVIT  
| | | | | | | | | | | | | | | | | | | | | |  
458 GTFDLSGIPPAPRGVPQIEVTFDILDANGILsVSAEEKGTGKRNQIVIT  
| | | | | | | | | | | | | | | | | | | | | |  
457 GKFEltGIPPAPRGVPQIEVTFDIDANGILNVSAVdKSTGKenKETIT  
| | | | | | | | | | | | | | | | | | | | | |  
458 GKFElsGIPPAPRGVPQIEVTFDIDANGILNVSAVeKSsGKqNKITIT  
| | | | | | | | | | | | | | | | | | | | | |  
456 GrFElsGIPPAP GVPQIEVTFDIDANGILNVtAtDKSTGKaNKITIT  
| | | | | | | | | | | | | | | | | | | | | |  
460 GKFdltGIPPAPRGVPQIEVTFDIDANGILNVSAvDKSTGKenKITIT  
| | | | | | | | | | | | | | | | | | | | | |  
459 GKFElsGIPPAPRGVPQItVTFDIDvNnILNVSAeDKtTGqkNKITIT  
| | | | | | | | | | | | | | | | | | | | | |  
437 GKFElsGIPPAPRGtPQIeVTFDIDaNgILNVSAvDKgTGkqNKITIT

FIGURE 2-11

473	IK	ntST LSeeEInkMiQE	EENreAD	alKkdK
471	IK	SSTGLSdDEIdnMVKEA	EENAdAD	KqRK
501	IKA	SS GLneDEIQkMVrDA	EaNAeAD	RK
505	NDKGRLSKADIERMVSeAAKYEsqDKeQrerIDA		KNGLENYAFSv	
507	NDKGRLSKADIERMVSdAAKYEAEDK ahvIDA		KNGLENYAFSM	
505	NDKFRLSKEDIERMVQEAEKYKAED		ekQRdkVssKNsLESYAFNM	
506	NDKFRLSKEDIEkMVQEAEKYKAdD		daQRERVdAKNALESYAFNl	
506	NDKGRLSKEeIERMVQEAEKYKAED		EvQRERVsAKNALESYAFNM	
508	NDKGRLSKddIdRMVQEAEKYKAED		EanRdRVgAKNsLESYtYNM	
507	NDKGRLSKEEIEkMVQEAEKYKAED		EevkkkVdAKNaLEnYaYNM	
585	NDKGRLSKEEIErMVadAdKYKAED		EkgrdrVsAKNsLE syvyt	

17/32

FIGURE 2-12

```

505 iEttVraeglinQL      EKsitDqgeK                      idpkqkellekq
      | |           ||      || | |
501 EE VelRNeadQLvfttEKtLkDlegKVEEA                      evtkanea
      || | ||           ||||
530 fEElVqtRN          qgdhllhstrkqVEEA      gdklpaddKtaiEsaltaL
                                   |||| | | | |
552 KNTvNePNVAGK      ieeADKNTiTSAVEEALqWLNhNQEASKEEYEHROKEL
      ||| | |||||      |||| | ||||| ||| ||||| ||| |||||
549 KNTiNDPNVAGK      ldDADKNvTtAVEEALrWLNdNQEASLEEEYnHROKEL
      | | | ||      | || | | | | || | || | || | || |
550 KaTVEDEklqGKI      nDEDKqkIldKCnEiISWLdkNQTAEEKEEfeHQOKEL
      | |||| ||      |||| | || ||| || ||||| ||| |
551 KSmVEDEnvKGKI      SDEDKrtIseKCTqVISWLenNQLAEKEEYafQOKdL
      || |||| ||||      | || || || | ||| | |||| | |
552 KSAVEDEgLGKI      SeaDKkKVLDKCQEVISWLDaNtLAEKdEfeHKKrKEL
      | |||| ||||      | || ||||| |||| | || | ||| |||
556 KqtVEDEKLKGKI      SdqDKqKVLDKCQEVISsLDrNQmAEEKEEyEHKqKEL
              ||      || | | | | || | || | || | || |
555      rntikddKIasklpaeDKkKiEDavdgaISWLDSNQLAEvEEfEdKmKEL
              ||      | || |||| | || | || | ||
529 mkqqvegelkeKIpesdhqviisKcED      tISWLDvhQsAEkhEyesKreEL

```

18/32

FIGURE 2-13

541	iqeLK	DL	lkedktDEL	kLkldqieaaaqsFAQa
539	kdALKaaiekndLeeIkAK		kDELgeivqaLtvKL	yeqAQ
573	EtALK	geDkaaIeAKM	qEL	aqvsqKlmeiaqqqhAQ
602	E	nlCtPImtKM	YQGMGaGGgmPG	GMPgG
599	E	gVCaPIIsKM	YQGMG GGdgPG	GMPeG
600	E	KVCnPIITK	LYQ	saG
601	E	KVCqPIITK	LYQ	G
599	E	qVCNPII	sgLYQ	GAG
603	E	kLCNPI	vtkLYQ	GAG
602	E	giCNPI	IakmyxgeGAG	
579	E	kvCaPI	I tkdvyqagG	



19/32

FIGURE 2-14

```

574 tAQQA                               ntsEsdpkaDDsntiDAEikqd
      ||||                               |      ||      |||
578 QAQA  G                               EggAqnDD  VVDAEEFEEVndDKK
      |||||  |                               ||      |||||  |||
609 Q QtA  G A                           daSAnnakdDD  VVDAEEFEEV kDKK
      |  |                               ||                               |
630 MPGGMPGG AnPssssgpkwrkwteSASlkmfpwrrllanE
      |||||  |                               ||
626 MPGGMPGG  mPG  G mgggmGGaaASSGPkvEEVD
      ||||  ||  |  ||  ||||  ||||
621 MPGG      fPG  GGA ppsGG  ASSGPTIEEVD
      |||  ||  ||  ||  |||||
620 vPGG      mPGsscGAQarqGG  nSGPTIEEVD
      |||  |||  ||  |||||
618 PGG      fGAQgpkGG  SGSGPTIEEVDO
      ||  ||  |||||
620      aGA      GG  SG GPTIEEVDO
      ||  ||  ||  |||||
620      MGAaaGM dedapsGG  SGaGPkIEEVDO
      |  ||  ||  ||  |||||
596      M  pgGMheasgagGG  SGkGPtIEEVD

```

FIGURE 4-1

EcoR1                      Kpn1

29  
 ARG ASP LEU GLY PRO ASP ARG CYS ARG GLY ASP ILE ALA ARG  
 CGA GAT CTC GGG CCC GAT CGA TGC CGC GGC GAT ATC GCT CGA  
 GCT CTA GAG CCC GGG CTA GCT ACG GCG CCG CTA TAG CGA GCT  
 -----  
 |  
 Xho1

43  
GLY SER leu  
  
GGA AGC ttg  
CCT TCG aac  
-----| |--  
          |          pUC18  
Hind3

# SUBSTITUTE SHEET

21/32

## FIGURE 4-2

by lower case letters; the nucleotide sequence of the pWHA148 synthetic oligonucleotide addition is designated by upper case letters. Numbers refer to the order of the expected amino acid sequence.

22/32

## FIGURE 5-1

Translation of *M. hyopneumoniae* 74.5kD Antigen Gene

```

1
met ala lys glu ile ile leu gly ile asp leu gly thr thr
ATG GCA AAA GAA ATC ATT TTA GGA ATC GAC CTT GGA ACA ACA
15
asn ser val val ala ile ile glu asn gln lys pro val val
AAC TCA GTT GTT GCA ATT ATT GAA AAT CAA AAA CCT GTC GTT
30
leu glu asn pro asn gly lys arg thr thr pro ser val val
CTC GAA AAT CCC AAC GGA AAA AGA ACA ACT CCA TCC GTT GTC
45
ala phe lys asn asn glu glu ile val gly asp ala ala lys
GCT TTT AAA AAC AAT GAA GAA ATT GTC GGG GAT GCA GCT AAA
60
arg gln leu glu thr asn pro glu ala ile ala ser ile lys
AGA CAA CTT GAA ACT AAC CCA GAA GCA ATC GCT TCA ATT AAA
75
arg leu met gly thr asp lys thr val arg ala asn glu arg
AGA TTA ATG GGA ACT GAT AAA ACA GTT CGT GCA AAT GAA AGA
90
asp tyr ile pro glu glu ile ser ala lys ile leu ala tyr
GAT TAT ATT CCT GAA GAA ATC TCG GCA AAA ATT CTT GCT TAT
105
leu lys glu tyr ala glu lys lys ile gly his lys val thr
TTA AAA GAA TAT GCT GAG AAA AAG ATT GGT CAT AAA GTA ACA
120
lys ala val ile thr val pro ala tyr phe asp asn ala gln
AAA GCA GTA ATT ACA GTA CCT GCT TAT TTT GAC AAT GCC CAA
135
arg glu ala thr lys asn ala gly lys ile ala gly leu gln
CGT GAG GCA ACA AAA AAT GCC GGA AAA ATC GCT GGA TTA CAA
150
val glu arg ile ile asn glu pro thr ala ala ala leu ala
GTA GAA AGA ATT ATA AAT GAA CCA ACA GCG GCC GCA CTT GCT
165
phe gly leu asp lys thr glu lys glu met lys val leu val
TTT GGC CTT GAT AAA ACT GAA AAA GAA ATG AAA GTT CTT GTC

```

SUBSTITUTE SHEET

FIGURE 5-2

180

tyr asp leu gly gly gly thr phe asp val ser val leu glu  
TAT GAC TTA GGT GGG GGA ACT TTT GAT GTC TCA GTT TTA GAA

195

leu ser gly gly thr phe glu val leu ser thr ser gly asp  
TTA TCC GGT GGA ACC TTC GAA GTT TTA TCA ACA AGT GGT GAT

210

asn his leu gly gly asp asp trp asp asn glu ile val asn  
AAT CAT TTA GGT GGG GAT GAC TGG GAT AAT GAA ATT GTA AAT

225

trp leu val lys lys ile lys glu val tyr asp phe asp pro  
TGA CTT GTT AAA AAA ATC AAA GAA GTA TAT GAT TTT GAT CCA

240

lys ser asp lys met ala leu thr arg leu lys glu glu ala  
AAA AGT GAT AAA ATG GCG CTT ACA AGA CTT AAA GAA GAG GCT

255

glu lys thr lys ile asn leu ser asn gln ser val ser thr  
GAA AAA ACC AAA ATT AAT CTT TCA AAT CAA AGT GTT TCT ACA

270

val ser leu pro phe leu gly met gly lys asn gly pro ile  
GTT TCT CTA CCA TTT TTA GGA ATG GGC AAA AAC GGG CCG ATT

285

asn val glu leu glu leu lys arg ser glu phe glu lys met  
AAC GTT GAA CTT GAA CTT AAA AGA TCA GAA TTT GAA AAA ATG

300

thr ala his leu ile asp arg thr arg lys pro ile val asp  
ACT GCC CAT TTA ATC GAT AGA ACT CGC AAA CCA ATT GTT GAT

315

ala leu lys gln ala lys ile glu ala ser asp leu asp glu  
GCT CTA AAA CAA GCA AAA ATT GAG GCT TCA GAT CTT GAT GAA

330

val leu leu val gly gly ser thr arg met pro ala val gln  
GTT CTC CTT GTA GGT GGA TCA ACA AGA ATG CCA GCT GTT CAG

345

ser met ile glu his thr leu asn lys lys pro asn arg ser  
TCA ATG ATT GAG CAT ACT TTA AAT AAA AAG CCA AAT CGT TCA

FIGURE 5-3

360

ile asn pro asp glu val val ala ile gly ala ala ile gln  
ATT AAT CCT GAT GAG GTA GTC GCA ATT GGT GCT GCA ATT CAA

375

gly gly val leu ala gly glu ile ser asp val leu leu leu  
GGG GGG GTT CTA GCT GGA GAG ATC AGT GAT GTT CTA CTT TTA

390

asp val thr pro leu thr leu gly ile glu thr leu gly gly  
GAT GTT ACT CCT TTA ACT TTA GGA ATT GAA ACT TTA GGT GGA

405

ile ala thr pro leu ile pro arg asn thr thr ile pro val  
ATT GCA ACA CCT TTG ATT CCA AGA AAT ACA ACA ATT CCG GTA

420

thr lys ser gln ile phe ser thr ala glu asp asn gln thr  
ACA AAA TCA CAA ATT TTC TCA ACA GCT GAG GAT AAT CAA ACC

435

glu val thr ile ser val val gln gly glu arg gln leu ala  
GAA GTA ACA ATT TCT GTT GTC CAA GGT GAA CGT CAA CTT GCA

450

ala asp asn lys met leu gly arg phe asn leu ser gly ile  
GCG GAT AAT AAA ATG TTA GGT CGC TTT AAT TTA TCA GGA ATT

465

glu ala ala pro arg gly leu pro gln ile glu val ser phe  
GAA GCT GCT CCA CGA GGT CTT CCC CAG ATT GAA GTT AGT TTT

480

ser ile asp val asn gly ile thr thr val ser ala lys asp  
TCA ATT GAT GTC AAC GGG ATT ACA ACG GTT TCA GCA AAA GAT

495

lys lys thr gly lys glu gln thr ile thr ile lys asn thr  
AAA AAA ACC GGC AAA GAA CAA ACA ATT ACA ATT AAA AAT ACT

510

ser thr leu ser glu glu glu ile asn lys met ile gln glu  
TCA ACT TTA TCA GAA GAA GAA ATT AAT AAG ATG ATT CAG GAA

525

ala glu glu asn arg glu ala asp ala leu lys lys asp lys  
GCC GAA GAA AAT CGT GAA GCT GAT GCT CTT AAA AAA GAC AAA

25/32

FIGURE 5-4

540

ile glu thr thr val arg ala glu gly leu ile asn gln leu  
ATC GAG ACA ACA GTT CGT GCC GAA GGG CTT ATT AAT CAA CTT

555

glu lys ser ile thr asp gln gly glu lys ile asp pro lys  
GAG AAA TCA ATA ACT GAT CAA GGT GAA AAA ATT GAT CCA AAA

570

gln lys glu leu leu glu lys gln ile gln glu leu lys asp  
CAA AAA GAA TTA CTT GAA AAA CAA ATT CAA GAA TTA AAA GAT

585

leu leu lys glu asp lys thr asp glu leu lys leu lys leu  
CTT CTA AAA GAA GAT AAA ACT GAC GAA TTA AAA TTA AAA TTA

600

asp gln ile glu ala ala ala gln ser phe ala gln ala thr  
GAC CAA ATT GAA GCA GCT GCC CAA TCT TTT GCG CAG GCA ACC

615

ala gln gln ala asn thr ser glu ser asp pro lys ala asp  
GCG CAG CAA GCA AAT ACA TCT GAA TCT GAT CCA AAA GCT GAT

630

asp ser asn thr ile asp ala glu ile lys gln asp OC  
GAT TCA AAC ACA ATT GAT GCT GAA ATC AAG CAG GAT TAA

FIGURE 11-1

Translation of *M. gallisepticum* 67 kD Antigen Gene

```

1
met ser asn asn asn gly leu ile ile gly ile asp leu gly
ATG TCT AAT AAT AAT GGA TTA ATT ATT GGA ATT GAT CTT GGT
15
thr thr asn ser cys val ser val met glu gly ala gln lys
ACC ACC AAC TCT TGT GTG TCT GTA ATG GAA GGT GCA CAA AAA
30
val val ile glu asn pro glu gly lys arg thr thr pro ser
GTA GTA ATT GAA AAC CCA GAA GGT AAA AGA ACT ACT CCA TCA
45
val val ser tyr lys asn gly glu ile ile val gly asp ala
GTA GTT TCA TAC AAA AAC GGT GAA ATT ATT GTT GGT GAT GCT
60
ala lys arg gln met leu thr asn pro asn thr ile val ser
GCT AAG CGT CAA ATG CTA ACT AAC CCA AAC ACT ATT GTT TCT
75
ile lys arg leu met gly thr ser lys lys val lys ile asn
ATT AAG CGT TTA ATG GGA ACA AGT AAA AAA GTT AAG ATT AAT
90
asp lys gly val glu lys glu leu thr pro glu glu val ser
GAC AAA GGT GTA GAA AAA GAA CTT ACT CCA GAA GAA GTT TCT
105
ala ser ile leu ser tyr leu lys asp tyr ala glu lys lys
GCT AGC ATC TTA AGT TAT CTT AAA GAT TAC GCT GAA AAG AAA
120
thr gly gln lys ile ser arg ala val ile thr val pro ala
ACT GGT CAA AAG ATT TCA AGA GCT GTA ATT ACT GTT CCA GCT
135
tyr phe asn asp ala glu arg gln ala thr lys thr ala gly
TAC TTC AAC GAC GCT GAA CGT CAA GCT ACT AAA ACT GCT GGT
150
lys ile ala gly leu thr val glu arg ile ile asn glu pro
AAG ATT GCT GGT TTA ACT GTA GAA AGA ATT ATT AAC GAA CCT

```



27/32

## FIGURE 11-2

165

thr ala ala ala leu ala tyr gly ile asp lys gly his arg  
ACA GCA GCT GCA TTA GCT TAT GGT ATT GAT AAA GGT CAC CGT

180

glu met lys val leu val tyr asp leu gly gly gly thr phe  
GAG ATG AAA GTT CTT GTG TAC GAC CTT GGT GGT GGT ACG TTT

195

asp val ser leu leu asp ile ala asp gly thr phe glu val  
GAC GTT TCA TTA CTT GAT ATT GCT GAT GGT ACT TTC GAA GTT

210

met ala thr ala gly asp asn arg leu gly gly asp asp trp  
ATG GCT ACT GCT GGT GAT AAC AGA TTA GGT GGT GAT GAC TGA

225

asp asn lys ile ile glu trp ile ile ala glu ile lys lys  
GAT AAT AAG ATT ATT GAA TGG ATC ATT GCT GAA ATC AAA AAA

240

asp his pro ser leu asp leu lys ser asp lys met ala met  
GAT CAC CCA TCA TTA GAC CTT AAG TCT GAT AAG ATG GCA ATG

255

gln arg leu lys glu ala ala glu arg ala lys ile glu leu  
CAA AGA TTA AAA GAA GCT GCT GAA AGA GCT AAG ATC GAA CTA

270

ser ala gln leu glu thr leu ile ser leu pro phe ile ala  
TCA GCT CAA TTA GAA ACA CTA ATC TCA TTA CCA TTC ATC GCA

285

val thr pro glu gly pro val asn ala glu leu thr leu ser  
GTT ACT CCT GAA GGT CCA GTA AAC GCT GAA TTA ACT TTA TCA

300

arg ala lys phe glu glu leu thr lys asp leu leu glu arg  
AGA GCT AAA TTC GAA GAA TTA ACT AAA GAC TTA CTA GAA AGA

315

thr arg asn pro ile ala asp val leu lys glu ala lys val  
ACA AGA AAC CCA ATT GCT GAC GTA TTA AAA GAA GCT AAG GTT

FIGURE 11-3

330

asp pro ser gln val asp glu ile leu leu val gly gly ser  
GAT CCT AGT CAA GTT GAT GAA ATT CTT TTA GTA GGT GGT TCT

345

thr arg met pro ala val gln lys leu val glu ser met ile  
ACA AGA ATG CCT GCA GTA CAA AAA TTA GTT GAA TCA ATG ATT

360

pro asn lys ala pro asn arg thr ile asn pro asp glu val  
CCT AAT AAA GCA CCA AAC CGT ACG ATT AAC CCT GAC GAA GTA

375

val ala ile gly ala ala val gln gly gly val leu arg gly  
GTA GCG ATC GGT GCT GCT GTA CAA GGT GGG GTA TTA CGT GGG

390

asp val lys asp ile leu leu leu asp val thr pro leu thr  
GAT GTT AAA GAC ATC TTA TTA TTA GAC GTA ACT CCT TTA ACA

405

leu ala ile glu thr leu gly gly val ala thr pro ile ile  
CTT GCG ATT GAA ACT TTA GGA GGT GTA GCA ACT CCA ATT ATT

420

lys arg asn thr thr ile pro val ser lys ser gln ile phe  
AAG AGA AAC ACA ACT ATT CCA GTT TCT AAA TCA CAA ATC TTC

435

ser thr ala gln asp asn gln glu ser val asp val ser ile  
TCA ACA GCT CAA GAT AAC CAA GAA TCA GTT GAC GTT TCA ATT

450

tyr gln gly glu arg pro met ala arg glu asn lys ser leu  
TAC CAA GGT GAA CGT CCA ATG GCT AGA GAA AAC AAA TCA TTA

465

gly thr phe ser leu gly gly ile gln pro ala pro lys gly  
GGA ACT TTC TCA CTT GGA GGA ATT CAA CCA GCT CCT AAG GGT

480

lys pro gln ile glu ile thr phe asn ile asp ala asn gly  
AAA CCA CAA ATT GAA ATT ACT TTC AAT ATT GAC GCT AAC GGG

29/32

FIGURE 11-4

495

ile leu asn val lys ala lys asp leu thr thr gly lys glu  
ATT TTA AAT GTT AAG GCT AAA GAC TTA ACA ACT GGT AAA GAA

510

asn ser ile thr ile ser asn ser ser glu leu asp glu asn  
AAC AGT ATT ACG ATC TCT AAC TCA AGT GAA TTG GAT GAA AAC

525

glu ile gln arg met ile arg asp ala glu ala asn lys glu  
GAA ATC CAA AGA ATG ATC CGT GAT GCT GAA GCT AAC AAA GAA

540

arg asp ala ile val lys gln arg ile glu met arg tyr glu  
CGT GAC GCA ATC GTT AAA CAA AGA ATC GAA ATG CGT TAT GAA

555

gly glu gly ile val asn thr ile asn glu ile leu gly ser  
GGT GAA GGA ATT GTT AAT ACA ATT AAC GAA ATC CTT GGT TCT

570

lys glu ala glu ala leu pro ala gln glu lys ala ser leu  
AAA GAA GCA GAA GCG CTA CCT GCT CAA GAA AAA GCT AGC CTT

585

thr lys ile val asp gly ile asn gly ala leu lys ala glu  
ACT AAG ATC GTT GAT GGA ATT AAC GGT GCT CTT AAA GCT GAA

600

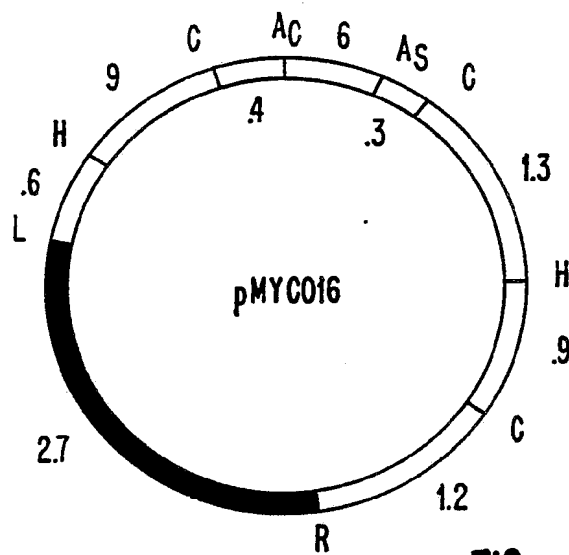
lys trp asp glu leu lys glu gln ile asp gly phe lys lys  
AAA TGA GAT GAA CTT AAA GAA CAG ATC GAC GGC TTC AAG AAA

615

trp arg asp asp met ser lys lys tyr gly gly gly glu ala  
TGA CGT GAT GAC ATG TCT AAG AAA TAC GGT GGT GGC GAA GCT

630

pro ala glu pro lys AM  
CCA GCC GAA CCT AAA TAG

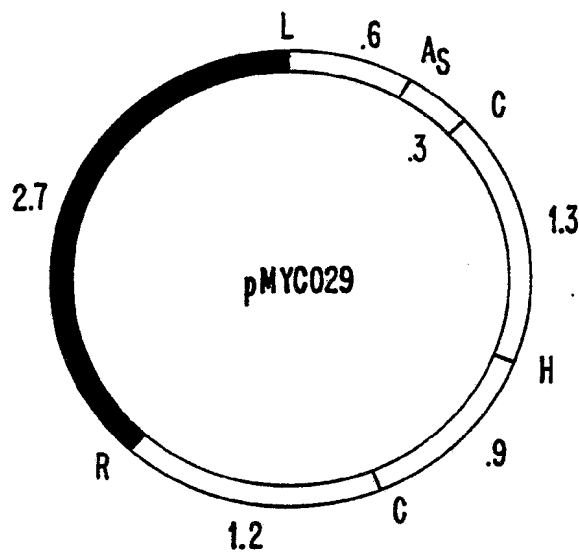


## LEGEND

AC AccI  
 AS AsuII  
 C ClaI  
 H HindIII  
 L HindIII-ClaI-PstI-AccI-EcoRI  
 R EcoRI

■ pWHA148  
 □ MYCOPLASMA

FIG. 3.

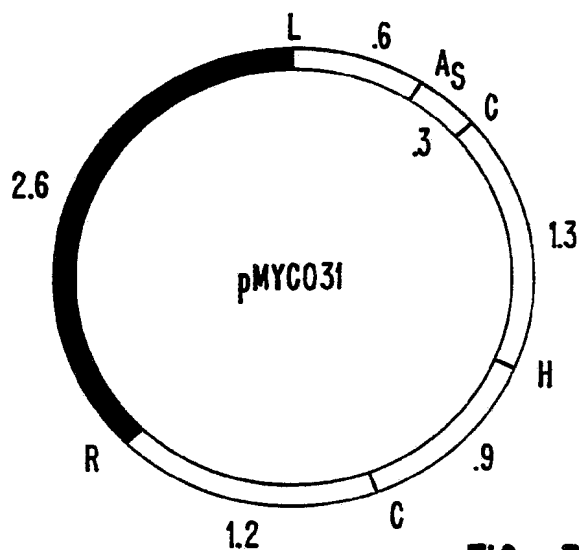


## LEGEND

AS AsuII  
 C ClaI  
 H HindIII  
 L HindIII-ClaI-PstI  
 R EcoRI

■ pWHA148  
 □ MYCOPLASMA

FIG. 6.



## LEGEND

AS AsuII  
 C ClaI  
 H HindIII  
 L HindIII-PstI  
 R EcoRI

■ pUC9  
 □ MYCOPLASMA

FIG. 7.

31/32

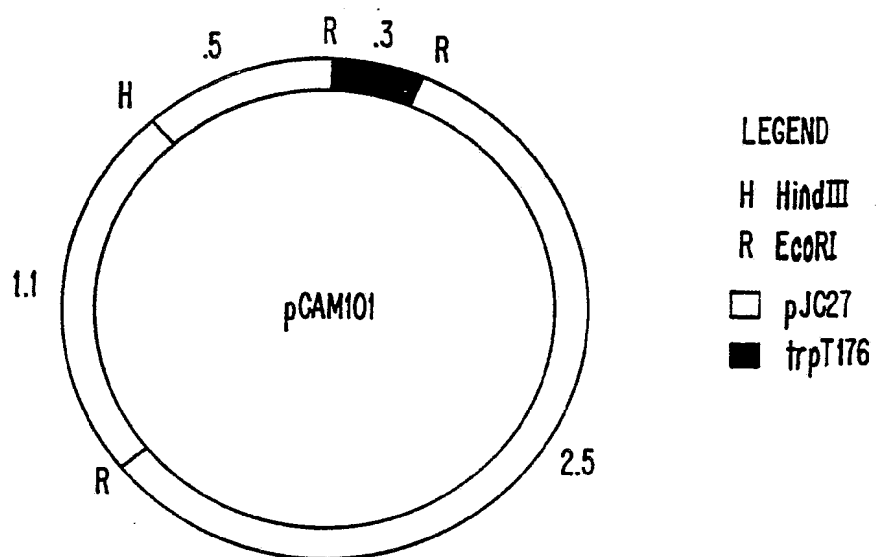


FIG.\_8.

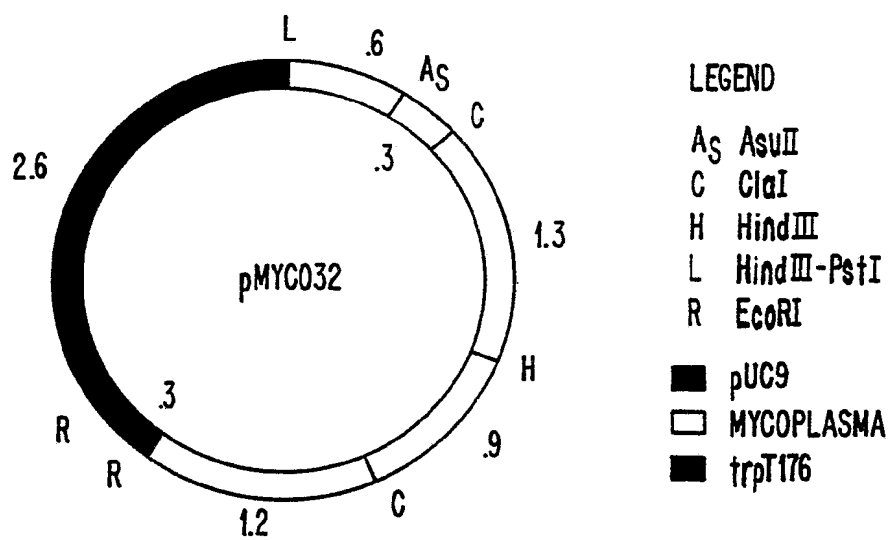


FIG.\_9.

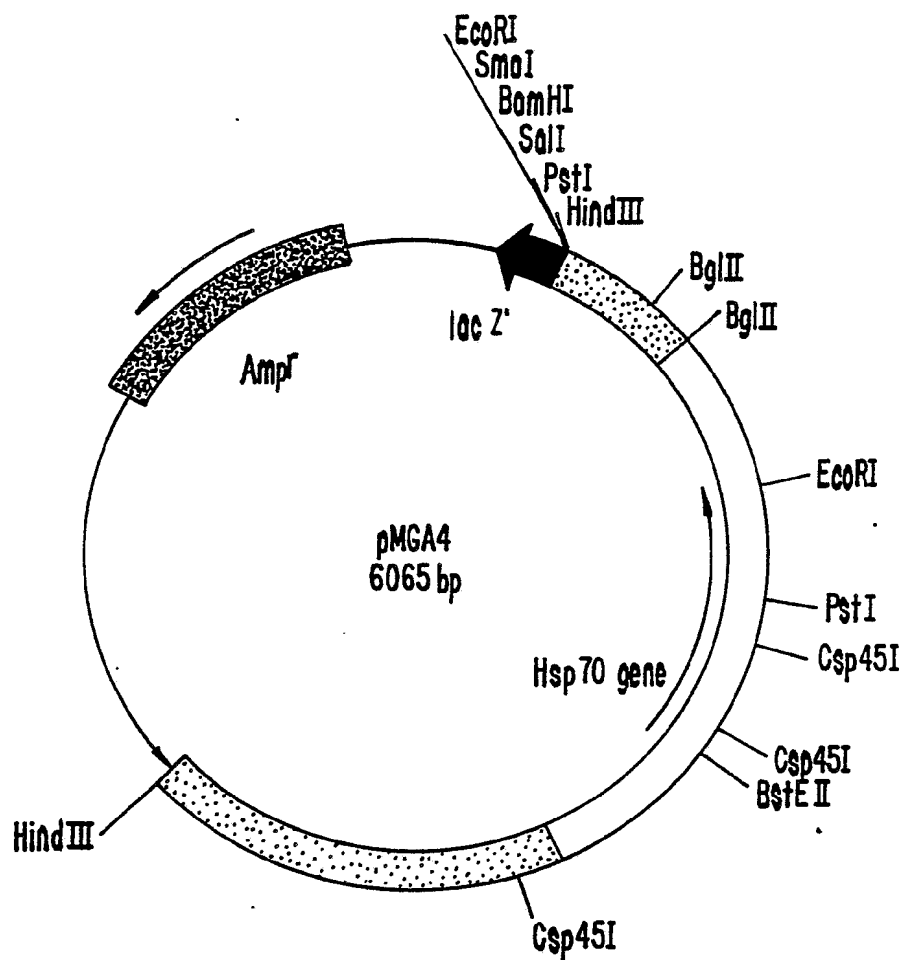


FIG. 10.

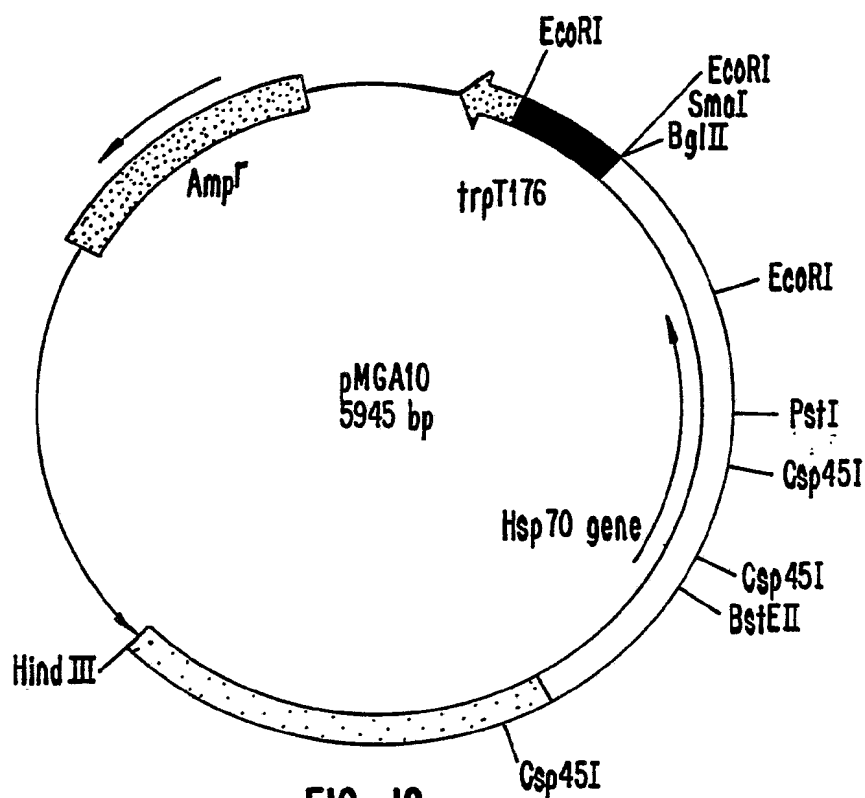


FIG. 12.

# INTERNATIONAL SEARCH REPORT

International Application No. PCT/US89/03955

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (if several classification symbols apply, indicate all) <sup>6</sup> According to International Patent Classification (IPC) or to both National Classification and IPC <b>IPC(4): A61K39/005, 39/04, 39/40; C12N15/00, 1/00; C12P21/00; G01N33/53; A61K39/395</b>		
<b>II. FIELDS SEARCHED</b>		
Minimum Documentation Searched <sup>7</sup>		
Classification System	Classification Symbols	
U.S.	424/88; 435/7, 172.1; 536/27	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched <sup>8</sup>		
Databases: Chemical Abstracts Services Online (File CA, 1967-1989; File Biosis, 1969-1989). Automated Patent System (USPAT), 1975-1989). Sequence search (protein databases: PIR, Swiss-Prot).		
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT <sup>9</sup></b>		
Category <sup>*</sup>	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>
X Y	Molecular and Cellular Biology, Volume 6, Number 12, December 1986, Glass, "Conserved sequences and transcription of the hsp70 gene family in Trypanosoma brucei", pp. 4657-4666.	1-11, 17 12-16
X Y	Cell, Volume 2, October 1980, Ingolia, "Sequence of three copies of the gene for the major drosophila heat shock induced protein and their flanking regions", pp. 669-679.	1-11, 17 12-16
X Y	Nucleic Acids Research, Volume 15, Number 13, 1987, Dworniczak, "Structure and expression of a human gene coding for a 71 kd heat shock 'cognate' protein", pp. 5181-5197.	1-11, 17 12-16
Y	US, A, 3,993,743 (Hanson) 23 November 1976	12-16
Y	Phil. Trans. R. Soc. Lond., Volume B 307, 1984, Scott, "The vaccine potential of cell	1-17
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p><sup>*</sup> Special categories of cited documents: <sup>10</sup></p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&amp;" document member of the same patent family</p> </div> </div>		
<b>IV. CERTIFICATION</b>		
Date of the Actual Completion of the International Search		Date of Mailing of this International Search Report
08 December 1989		10 JAN 1990
International Searching Authority		Signature of Authorized Officer
ISA/US		D. Bernstein